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Molecular Biological Studies On The Rep Gene Of Escherichia Coli

Carol Anne Gilchrist

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MOLECULAR BIOLOGICAL STUDIES ON THE *rep* GENE OF *Escherichia coli*

BY

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ABSTRACT

My work has involved the sequencing of the Escherichia coli rep gene. This gene codes for the Rep helicase, a ssDNA-dependent ATPase required by some phages (ΦX174, fd, P2) for their replication.

The section of E. coli chromosome known to contain the rep gene, which I have sequenced, has only one transcribed open reading frame of the correct size to encode the Rep protein. The predicted N-terminal amino acids and the predicted total amino acid composition are in agreement with those determined from purified Rep protein.

The proposed amino acid sequence of Rep contains in it's N terminus a peptide common to all ATP-binding enzymes: $G/A-X_4-GKT-X_6-I$. It is also possible to use the primary amino acid sequence data to predict a secondary structure for the Rep protein. The amino acid sequence of the Rep protein was compared with others already present in the protein sequence data bank; DNA helicase II the uvrD gene product, was identified as the only protein with significant homology with Rep.

In order to correlate structural features with functional protein domains two approaches were used. The first approach involved creating minor deletions in the gene and assaying for some of Rep's activities.

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LIST OF ABBREVIATIONS

A	adenine
A	alanine
ADP	adenosine 5'-diphosphate
Ala	alanine
Ap	ampicillin
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
C	cysteine
cpm	counts per minute
Cys	cysteine
D	aspartic acid
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosaphate
ddCTP	dideoxycytidine 5'-triphosphate
ddGTP	dideoxyguanosine 5'-triphosphate
ddTTP	dideoxythymidine 5'-triphosphate
DEAE	diethylaminoethyl

DNA	deoxyribonucleic acid
dGTP	deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
E	glutamic acid
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
F	phenylalanine
g	gravity
G	guanine
G	glycine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
H	histidine
His	histidine
I	isoleucine
Ile	isoleucine
IPTG	isopropyl ß-D-thio-galactopyranoside
K	lysine
kb	kilobase
kdal	kilodalton
klenow	E. coli DNA polymerase I-klenow fragment
Km	kanamycin

L	leucine
Leu	leucine
Lys	lysine
M	methionine
Met	methionine
moi	multiplicity of infection
Mr	molecular weight
mRNA	messenger ribonucleic acid
N	asparagine
NEN	New England Nuclear
P	proline
PBS	Phosphate Buffered Saline
PEG	polyethylene glycol
pfu	plaque forming unit
Phe	phenylalanine
polI	E. coli DNA polymerase I
Pro	proline
Q	glutamine
R	arginine
RNA	ribonucleic acid
S	serine
SDS	sodium dodecyl sulphate
Sequenase	modified T7 DNA polymerase
Ser	serine
ss	single-stranded

ssb	*E. coli* single-stranded DNA binding protein
SSC	standard saline citrate (NaCl 0.15M, Na citrate 15mM)
sup	suppressor
T	thymine
T	threonine
Tc	tetracycline
TBS	Tris-buffered saline
TE	Tris(HCl) 10mM, EDTA 1mM pH8
TEAB	triethylammonium bicarbonate pH 7.0
Thr	threonine
ts	temperature sensitive
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet light
V	valine
Val	valine
vol	volume
W	tryptophan
w.t.	wild type
Y	tyrosine

Chapter 1

Introduction

1.01 HELICASES

A protein is said to have a helicase activity if it is capable of utilizing energy to unwind double-stranded nucleic acid. In all systems so far studied a helicase activity is required for duplex DNA replication since the DNA synthesis machinery requires single-stranded DNA as a substrate. This is true in the eukaryotic systems studied, which include yeast, calf thymus (Valentini et al., 1984) rodent cells (Cobianchi et al., 1986), mouse myeloma and HeLa cells (Cobianchi et al., 1985) as well as in the prokaryotes. The best studied system is that of the prokaryote Escherichia coli.

1.02 Escherichia coli HELICASES

There are a number of proteins known to have a helicase activity present in E. coli (Kornberg, 1982; Abdel-Monem and Hoffmann-Berling, 1980; Taucher-Scholz and Hoffmann-Berling, 1983). In studying Rep it is necessary, for a clear comprehension of Rep's function in vivo, to understand what is known about the other helicases and to relate the role that they play in E. coli metabolism with what is known about Rep's role. To this end a brief description of the helicase proteins known to be present in E. coli follows,

the helicases thought to be most important in the cell's metabolism being described in greater detail. How their activities interact, or substitute, for Rep is described in the next section on the Rep helicase and its role in E. coli.

1.03 DNA Helicase I

The DNA helicase I protein, which has a molecular weight of 180,000, was the first protein containing a DNA unwinding activity identified. This helicase requires a 20-base pair single-stranded DNA gap in the double-stranded helix to initiate unwinding. Between 70 to 80 protein monomers bind to this region and then proceed processively in a 5' to 3' manner through the DNA helix in a process that requires ATP. The DNA helicase I protein, which is present at about 500 to 700 copies per cell (Klinkert et al., 1980), is not encoded by the E. coli genome but by the F plasmid's traI gene (Abdel-Monem et al., 1983).

This helicase therefore may have a role in this plasmid's life cycle, probably at the stage of plasmid conjugation. As traI is a plasmid gene and E. coli replication proceeds normally in F⁻ cells, this helicase plays no role in normal cell metabolism (Taucher-Scholz and Hoffmann-Berling, 1983).

1.04 DNA Helicase II

This is a globular protein with a calculated molecular weight of 82,000 daltons (Finch and Emmerson, 1984). This protein has considerable homology with the Rep helicase.

DNA helicase II requires a 12-bp gap of single-stranded DNA in the double-stranded DNA helix to initiate unwinding (Abdel-Monem and Hoffmann-Berling, 1980). The helicase which has a strong affinity for single-stranded DNA binds to this gap. It was previously thought that DNA helicase II acts in a stoichiometric manner, however it has been found that partial duplexes which are either circular or retain a long stretch of single-stranded DNA to the 3' of the duplex region are the best substrates for this enzyme. This suggests that the protein may move in a processive manner along the single-stranded DNA (Matson, 1986).

DNA helicase II is present at a level of 5000 to 8000 copies per cell (Klinkert et al., 1980; Abdel-Monem, and Hoffmann-Berling, 1980) This helicase unwinds in a 3' to 5' direction, not the 5' to 3 direction originally thought; the experiments preformed by S: Matson utilizing partial double-stranded DNA show this clearly. A partial duplex was created by hybridizing a 341-bp fragment to single-stranded M13. This molecule was cut in the double-stranded portion. The resulting linear molecule had a double-stranded segment of 200 bp at the 3' end and after an intervening single-

stranded DNA section had a double-stranded region of 143 bp at the 5' end. The addition of DNA helicase II led to the displacement of the 143-bp fragment indicating therefore that the unwinding is proceeding in a 3' direction (Matson, 1986).

DNA helicase II is capable of unwinding the DNA helix at a rate of 1000 base pairs per second. This is comparable with the rate of progression of the DNA replication fork in E. coli. There is also some preliminary antibody evidence that this protein is present at the replication fork (Klinkert et al., 1980), raising the possibility that this helicase is involved in unwinding the helix in DNA replication.

The gene which encodes DNA helicase II, uvrD (Arthur et al., 1982 Maples and Kushner, 1982), is present on the E. coli chromosome at 85 minutes. This gene was originally identified due to the fact that uvrD⁻ mutants are repair defective. The UvrD protein is not essential for normal DNA replication as uvrD⁻ strains can grow normally. This is not what would be expected of a protein with the role of unwinding the DNA helix ahead of the replication fork machinery.

The sensitivity of uvrD mutants to UV or other DNA damaging agents suggests that DNA helicase II plays a role in the DNA repair system in E. coli. A model proposed suggests that the uvrA, uvrB and uvrC genes are involved in

identifying a lesion in the DNA and introducing two nicks in the bordering DNA. The unwinding activity of DNA helicase II in concert with DNA polymerase I leads to the dissociation of the mutated DNA and turnover of the UvrA, B, C proteins. The DNA polymerase I protein resynthesizes the DNA to produce a new daughter strand which is non-mutagenic (Caron et al., 1985, Hasain et al., 1985). There are two lines of evidence which support this model.

The addition of DNA helicase II into an in vitro repair system, which contained UV-irradiated DNA, UvrA, UvrB, UvrC proteins, DNA polymerase I, and dNTP's, increased the rate of repair enzyme turnover to in vivo levels. This increased turnover rate permitted greater identification and nicking at lesions in the DNA and thus a concomitant increase in repair resynthesis of the DNA.

The second line of evidence lies in the fact that uvrD's transcription appears to be co-regulated with that of the excision repair genes by the SOS system. These genes are normally downregulated by the binding to their promoter of the constitutively produced LexA protein. On DNA damage this protein is cleaved by the RecA protein leading to the increased production of the repair enzymes (Brandsma et al., 1983).

Experiments utilizing the uvrD promoter show that there is reduced in vitro transcription when LexA protein is added, and DNAse I protection assays indicate a region

downstream of the promoter is protected on LexA binding. When this area was sequenced it was shown to have considerable homology to other known LexA binding sites for which a consensus SOS box sequence CAG-X_{10}-CTG has been identified (Easton and Kushner, 1983).

DNA helicase II therefore seems to play a role in excision repair and may have an involvement in DNA replication.

1.05 DNA Helicase III

This helicase has not been as well characterized as the others as the loss of this helicase appears to have no known effect on E. coli metabolism.

Known facts include its size, 56,000 Mr, and that it is present in only 20 copies per cell and has a requirement for ATP for its helicase activity, which is 5' to 3' in direction. This enzyme requires single-stranded DNA binding protein to prevent strand reannealing after the helicase has passed through the DNA helix as it acts by a stoichiometric mechanism (Yarranton et al., 1979a; Yarranton et al., 1979b).

1.06 Rho

The Rho protein is essential for cell viability and has a molecular weight of 46,094 Mr (Pinkham and Platt, 1983). It is active in its hexameric configuration in which it has an ATPase activity and a ability to bind to poly(C). It has been identified as a transcription termination factor important in the correct termination of some RNA transcripts; for example at the termination site, t', of the trp operon (Christie et al., 1981). Few specific signals have been identified that indicate where Rho-dependent termination will occur. It is known that Rho binding is favoured in cases where the RNA has a high poly(C) content and has an untranslated stretch of at least 50 nucleotides (Glass, 1982; Bear et al., 1985).

The mechanism by which Rho achieves transcription termination was clarified by the discovery of an ATP-requiring helicase activity in the Rho protein.

This helicase activity is present in the aggregated hexameric complex which binds to RNA, presumably via the signals discussed above, and then moves through the RNA-DNA duplex in a 5' to 3' direction relative to the RNA in a process which requires ATP (Bennan et al., 1987) . The unwinding is thought to decrease the stability of the RNA polymerase/DNA complex leading to its dissociation and the release of the RNA from its DNA template.

1.07 **DnaB**

This protein is, like Rho, essential for E. coli viability. It is 52,268 Mr and encoded by the dnaB gene at 91 min on the E. coli chromosome (Nakayama et al., 1984). It is present in roughly 20 copies per cell and is active as a hexamer.

Mutations in this gene are known to interfere both with the process of initiating rounds of replication (the slow stop phenotype) and in the continuing process of elongation at the replication forks (the fast stop phenotype)(Kornberg, 1980; Kornberg, 1982).

The DnaB protein's role in initiation of replication has been examined by work on in vitro systems utilizing the oriC-containing plasmids. In these systems it was discovered that one of the earliest stages in initiation, prepriming, involves the formation of a F1* complex. The formation of this complex requires the oriC DNA sequence and ATP besides the presence of the proteins DnaA, DnaC and DnaB. The F1* complex exhibits altered gel mobility characteristics and S1 evidence indicates that this altered mobility is due to an increase in the quantity

of single-stranded DNA present i.e. the formation of this complex requires a helicase-like activity. The unwinding activity is known to utilize NTP's as an energy source and DnaB is the only protein present which contains a ssDNA-dependent NTPase activity. The DnaB protein hexamer can bind to single-stranded DNA.

The order of reactions at the origin appears to be first the binding of the DnaA protein to oriC, specifically to the oriC R sequences which are homologous to the consensus DnaA binding sequence TTAT(C/A)CA(C/A)A. To this then binds the preformed DnaB/DnaC complex. The DnaB protein then presumably unwinds the double-stranded DNA helix using NTP's to present single-stranded DNA substrate for the rest of the replication machinery (Baker et al., 1986). It appears probable that DnaB is the initiation machinery helicase.

As mentioned earlier this protein is known also to be present and essential at the elongating replication fork where it is thought to be involved in the synthesis of RNA primers for discontinuous DNA synthesis. Its presence in the replicating fork and its helicase activity suggest that it may have the role of unwinding the DNA helix at that site also.

Given the low copy number of this protein, and it's ATP/GTP dependence it is likely that the DnaB helicase's mode of action is processive like that of the primosome (n', DnaB) (Kornberg, 1982).

1.08 Rep Helicase

The rep helicase has a Mr. of 72,800 encoded by the rep gene at 83.5 min on the E. coli genome. It is a ssDNA-dependent ATPase utilizing two ATP molecules to unwind 1 base pair of double-stranded DNA helix (Kornberg et al., 1978). The Rep helicase is present in the cell at low levels (50 copies per cell) and acts in a processive manner. It proceeds through the helix in the 3' to 5' direction, requiring single-stranded DNA binding protein to prevent strand reannealing. This was shown by Yarrington and Gefter (1979) who created partial duplexes of ΦX174. These were asymmetrically cut and used as substrate for the Rep helicase. The quantity of double-stranded DNA remaining after the action of Rep was determined by S1 nuclease digestion followed by TCA (trichloroacetic acid) precipitation. When the substrate had a large duplex area to the 3'end of the intervening single-stranded DNA and had only a minor quantity of duplex DNA to the 5'end little double-stranded DNA remained to be precipitated by TCA. The helicase activity of Rep had unwound the majority of the double-stranded DNA indicating that Rep's helicase activity proceeds in a 3' to 5' direction.

Mutations in the rep gene were first isolated on the basis of the failure of certain phages to grow on these strains. The phages in question were the icosahedral phages

such ΦX174, G4, S13 the filamentous phages f1, fd, M13 and the noninducable temperate phages P2 and 186 (Takahashi et. al., 1978) .

In the case of ΦX174 it was observed that there were two phenotypes of the rep mutations: One which interferes with ΦX174 RFI replication and the second which blocked the production of single-stranded ΦX174 DNA for packaging into virions (Iwaya, 1971). In an in vitro ΦX174 RFI replication system it is necessary for the ΦX174 A protein and Rep to be present before DNA replication can occur. The second class of mutations, designated groL or repB, interfere with the production of single-stranded progeny. In vitro this step requires not only the presence of the ΦX174 A protein and the Rep helicase but also the phage-encoded F protein (Tessman and Peterson 1976; Eisenberg et al., 1976).

Rep can initiate unwinding at a nick in the double-stranded DNA helix (Kornberg et al., 1978) and thus it is essential for phages which use only a nick in their DNA to initiate DNA unwinding for phage replication.

The Rep helicase, although essential for these phages replication, is not essential for E. coli growth. The rep strains are not altered in their growth rate. There is, however, an alteration in the cells metabolism in regard to DNA replication and a slightly increased

sensitivity to DNA-damaging reagents (Denhardt et al., 1972; Bridges, 1984).

In rep mutants, although the overall quantity of DNA replication is the same there is a doubling of the rate of initiation, perhaps to compensate for a decrease in the rate of progression of the replicating forks (Lane and Denhardt, 1975; Colasanti and Denhardt, 1987).

What role, if any, Rep plays in the initiation process is not clear. The only suggestion that the DnaB helicase is not the only one present at oriC is the isolation of the dasC mutations, to be discussed later in this thesis, and the observation noted above that there seems to be some feedback between an alteration in replication fork progression resulting from the loss of the Rep protein function and the timing of initiation.

This decrease in the apparent average rate of elongation at the replication fork suggests that Rep has a role in unwinding the double-stranded DNA helix ahead of the replication machinery. The cell does not display an absolute requirement for the Rep protein, and there are other proteins which are candidates for this role; as mentioned earlier the DnaB protein is known to have a helicase activity and is essential for replication fork progression. Some antibody evidence indicates that the DNA helicase II protein is also present at the replication fork. It is therefore possible that all these have a backup

role in regard to each other. If one of these proteins is lost the other helicases can substitute for its activity at the replication fork.

In regard to the above theory it is interesting that a combination of a rep mutation and a mutation in the DNA helicase II gene uvrD has been suggested to be lethal to the cell (Taucher-Scholz et al., 1983). The Rep and DNA helicase II proteins have considerable similarity to each other, not only on the amino acid level, where they are roughly 30% similar, but in their directionality and size.

It is possible that it is the failure of another backup system that is responsible for the lethality of the combination of some rho mutations with a rep mutant. As mentioned earlier in this text Rho is an essential protein in E. coli, but there are mutations in rho which have a less severe phenotype. The Rho protein appears to be important not only as a transcription termination factor but also in DNA repair.

When the rho-15 allele, which is only defective in transcription termination, is combined with a rep mutant, cell death occurs. As mentioned earlier, rep mutations interfere with the cells' ability to survive DNA-damaging reagents. It is possible that Rho helicase activity and the Rep's helicase can substitute for each other to a degree in this process (Fassler et al., 1985).

Rep's role in DNA repair is still not totally known but it does not seem to be involved in the SOS response. The combination of a rep allele with a lexA constitutive strain leads to a further decrease in U.V. survival of approximately 20%. This result indicates that these two mutations are not lesions in the same repair pathway. Futhermore the induction of the SOS response leads at most to a two-fold increase in Rep production (Białkowska-Hobrzanska et al., 1985). Similarly no SOS box (the site of LexA binding in genes controlled by the SOS pathway) has been found in the rep promoter sequence, whereas it is present in the uvrD promoter and other genes controlled by LexA.

Some evidence exists that Rep is involved in recombination repair. B.A. Bridges (1984) used substrate DNA with high levels of crosslinks (induced by treating the cells with 8-methoxypsoralen and then exposing them to near UV light). The mechanism by which these DNA lesions are repaired is less dependent on the excision repair pathway and more dependent on recombination repair. As seen in the survival of treated cells which have mutations in the uvrA gene versus recA$^-$. The survival of rep strains after 8-methoxy-psoralen treatment decreased further from uvrA strain levels. When the rep-3 mutant is combined with a recA mutant, no further decrease in repair efficiency is observed indicating that the recA gene and rep are involved in the same repair pathway.

Another indication that rep is involved in recombination repair is derived from experiments by J. Zeig et al., (1978). They compared the rate of recombination between two different tandemly arrayed lac deletions in the trans configuration to reconstitute the lac operon in rep$^+$ and rep$^-$ strains and found it is decreased in rep$^-$ strains as well as in recA mutants.

A model for Rep's role may be provided by the T4 phage. In this phage a recA-like activity, uvsX, initiates crossover between sister strands. It has been discovered that once the crossover event has occurred, the rate of strand exchange is greatly increased if the T4 helicase dda is added. In contrast, the addition of dda to the recA system has no effect; this helicase effect is, therefore, specific to the T4 system and is not the result of adding any helicase to a strand exchange reaction (Kodadek and Alberts, 1987). If a similar effect could be achieved with the E. coli recA enzyme it would, like uvsX, probably have at least some degree of specificity for the helicase it would interact with. The linkage of rep with the recombination repair pathway it is a could act in a similar system in E. coli. Rep, then, would increase the rate of sister-strand crossover in recombination repair and its loss would decrease the effectivness of this repair pathway leading to

the observable phenotype of repair defectiveness in rep mutants.

Another lethal combination is that of some single-stranded binding protein mutants with rep alleles. Why this combination is lethal is not known. As Rep is a processive enzyme moving through the DNA helix it therefore requires, as mentioned earlier, the single-stranded DNA binding protein to prevent strand reannealling. However why the combination of ssb and rep is more deleterious than either alone is not clear. It is possible that the two proteins directly interact, which is supported by the fact that there are some rep mutants which suppress the ssb phenotype (Tessman and Peterson, 1982). This being the case there could be some mutant combinations which interfere with normal E. coli metabolism.

In summary then the Rep helicase may have a role at the replication fork to expedite strand elongation - a process in which it either acts in concert with, or can be substituted by, the helicases DnaB and/or DNA helicase II. Its loss appears to affect the timing of initiation of new rounds of DNA synthesis either through direct interaction or through a feedback mechanism resulting from the slowing of strand elongation. Its role in the repair of DNA damage seems to be in the process of recombination repair, where it may facilitate the rate of strand exchange once RecA has initiated crossover.

1.09 The aim of this thesis

The aim of this thesis is to achieve a greater understanding of the rep gene. I achieved this by using several different approaches.

1) Sequencing the E. coli DNA containing the rep gene.

The rep promoter was analyzed for the presence of sequences known to be important in gene regulation.

The identification of the major open reading frame downstream of this promoter enabled us to predict a possible primary and secondary structure for the Rep helicase. The amino acid sequence was then compared to that of other proteins.

2) Determination of transcription initiation and termination sites of the rep gene.

This not only confirmed the correctness of the promoter and open reading frame identification but also provided extra information about the rep promoter and permitted speculation on the nature of the termination signal.

3) The construction of rep mutants.

I created both a deletion and a minor insertion at the 'A' ATPase consensus sequence and assayed for

perturbation of Rep function. This allowed me to relate information about the amino acid sequence of Rep to its function.

4) Analysis of the dasC mutation.

As the dasC suppressor mutation resided in the vicinity of the rep gene we decided to investigate it. The dasC mutant phenotype appears to be due to the escape of the Rep protein from regulation by a neighboring DNA sequence. This suggests a possible regulated function of Rep in initiation of DNA replication in vivo.

CHAPTER 2

MATERIALS AND METHODS

2.01 Enzymes and Reagents

Restriction endonucleases were purchased from Bethesda Research Laboratories, Boehringer Mannheim or New England Biolabs; the Sequenase enzyme from the United States Biochemical Corporation; calf intestinal alkaline phosphatase, T4 polynucleotide kinase and T4 DNA ligase from Boehringer Mannheim; reverse transcriptase and M13 "17-mer" primer from New England Biolabs; the 8-base KpnI linker (CGGTACCG) was obtained from Collaborative Research Inc.. Elutip-d columns were from Schleicher and Schuell. All the above were used according to the manufacturer's directions. The large fragment of DNA polymerase I (DNA polymerase I-Klenow fragment) was purified from cells expressing it from the plasmid pCJ55 (Joyce and Grindley, 1983) by J. Colasanti and R. Khokha. The [α^{32}P]dATP (800 Ci/mmole) and [α^{35}S]dATP (500 Ci/mmole) were bought from New England Nuclear; unlabeled nucleotides as well as DEAE-cellulose were purchased from P-L Biochemicals (now Pharmacia P-L Biochemicals).

2.02 Bacterial Strains

The E. coli strains used in this thesis are detailed in Table 1. Escherichia coli MZ-1 was obtained from D. Court and was used to control expression of genes subcloned downstream of the lambda p_L promoter. E. coli JM103 was used to propagate M13 bacteriophages and E. coli Y1090 to propagate lambda. The TC-dasC strains were given to us by Dr. T. Atlung; the dnaA temperature-sensitive strains were given to us by E.B. Hansen or H.I. Eberle; and the temperature-sensitive tRNA dnaA(am) strains were provided by J. Shapiro. The E. coli dam strain Bu1255, given to us by G. Chaconas, allowed us to isolate DNA which could be cleaved by the methylation-sensitive restriction enzymes BclI and StuI.

2.03 Media

The E. coli strain RRI was grown in Luria (L) broth and L-agar as described by Miller (1972) (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl) the t.s. dnaA strains were grown in NY medium as described by von Meyenberg et al., (1982) (0.5% NaCl, 0.2% $MgCl_2$, 0.5% Bacto-yeast extract, 1% NZamine) NY medium was also used in the rep expression experiments . JM103 was grown in YT medium (0.8% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl). To

Table 1

Bacterial Strains	Genotype	Source
MZ-1	(His⁻UV⁻Bio⁻Thi⁻) lambda cIts857N	D. Court
JM103	[Δ(lac-pro)rspL endA sbcB15 hsdR4 supE /F'traD36 proAB lacI ᵠZΔM15]	J. Messing
RR1	F⁻hsdS20 (r_B^-,m_B^-)ara-14proA2 lacY1 galK2 rpsL20 (Sm^r)xyl-5mtl-1supE44,lambda⁻	F. Boliver
Bu1255	F⁻gal ara thr leu thi dam⁻dcm6 T1R T$_e^R$ Su⁺	G. Chaconas
C600	F⁻thi-1thr-1leuB6 lacY1 tonA21 supE4 lambda⁻	This lab
Y1090	(ΔlacU169 proA⁺ Δlon araD139 strA supF [trp22::Tn10](pMC9))	"
SurK	(C600)Δrep	J. Colisanti
TC152	K12(thi ara+1met3 his trp purE lac xyl tsx rspL tna ilvY)uph	M. Masters
TC182	(TC152)dnaA46	T. Atlung
TC861	(TC182)dasC1	"
TC862	(TC182)dasC2	"
PC5	thy leu dnaA5	H.I Eberle
CRT46	thy tnr leu ilv thi lacY mtl dnaA46	"
JC12390	thr-1leu-6ara-14proA2 lacY1 tsx-33 galK2 his-4 rspL-31 mtl-1 argE3 thi-1 sus⁻S13⁻lambda⁻dnaA508 tnaA300::Tn10⁻	"
CM732	[metE46 trp-3 his-4 thi-1 galK2 lacY1 orlac24 mtl-1 ara-9 tsx-3 ton-1 rspL8]	E.B. Hansen
CM782	(CM732)dnaA211 lambda⁻	"
CM2738	(CM732)dnaA604 lambda⁻	"
CM2740	(CM732)dnaA606 lambda⁻	"
CM744	(CM732)dnaA205 lambda⁻	"
CM748	(CM732)dnaA203 lambda⁻	"
CM2556	(CM732)dnaA167 lambda⁻	"
NS388	F⁻del(lac)bglR proA trp(am)his thi rspL tsx supF81(Ts)tna::Tn10 dnaA366(Am)	J. Schaus

ensure that the JM103 E. coli had retained the F plasmid the JM103 was periodically streaked out on minimal glucose agar plates (1.5% agar 1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, 0.02% $MgSO_4$, 0.2% glucose, 5 ng/ml thiamine HCl). Unless otherwise indicated cells to be rendered competent were grown in SOB media (2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$ $6H_2O$, 10 mM $MgSO_4$ $7H_2O$). When needed, kanamycin and carbenicillin were used at 50 and 100 µg per ml, respectively.

2.04 E. coli stocks

E. coli cells were inoculated into the appropriate medium and allowed to grow at a permissive temperature for 18 h. An equal volume of this culture was then added to 1 ml of 100% glycerol. These stocks of E. coli were frozen at -70°C.

2.05 Subcloning vectors

DNA was subcloned into M13mp8 and M13mp9 (Messing et al., 1981) or M13mp18 and M13mp19 (Yanisch-Perron et al., 1985) for the preparation of single-stranded DNA for sequencing. Wild type and dasCrep alleles were subcloned into either pBR322 (Bolivar et al., 1977) or the plasmids

Table 2

PLASMIDS	SIGNIFICANT FEATURES	SOURCE
pBR322	Amp^R, Tc^R	F. Bolivar
pSP64	Amp^R, polyclonal restriction site	D.A Melton
pSP65	"	"
pKC30	Amp^R, pL promoter	M. Rosenberg
pSY317	Kan^R, oriC	S. Yasuda & Y. Hirota
pJH113	[illegible]ter	P. Kuempel
pHBH40	Amp^R, Tc^R, rep	this lab
pRep0	Amp^R, pL promoter, rep structural gene	"
pGT26	Amp^R, Tc^R, uvrD	S. Matson
pSPrep(N-P)	wt rep plus 500bp downstream	this work
pSPrep	wt rep in pSP64	"
pSP861+	dasCrep1 and 900 bp upstream	"
pSP861▲border	dasCrep1 gene	"
pSP861+3	dasCrep1 and 800 bp downstream	"
pSPdasCrep1	rep isolated from TC861	"
pBR862+	dasCrep2 and 900 bp upstream	"
pBRdasCrep2	dasCrep2 subcloned in pBR322	"
pSPdasCrep2	rep isolated from TC862	"
pSP3-1rep	an internal rep deletion of 30 aa	"
pSP8-6rep	a minor insertion of rep	"

pSP65 and pSP64 (Melton et al., 1984). Other plasmids used as a source of various DNA's are detailed in Table 2. The plasmid pHBH30 (Bialkowska-Hobrzanska and Denhardt, 1984) was the source of the wild type rep allele; plasmid pSY317 was used as a source of E. coli origin DNA (Fuller et al., 1981), pJH113 was a source of terminus DNA (Hensen and Kuempel, 1985), and pGT26 a source of uvrD DNA (Taucher-Scholz and Hoffmann-Berling, 1983).

2.06 RFI and Plasmid DNA Preparation

For large scale preparations, plasmid DNA was purified from a lysozyme-Brij 58 cleared lysate. This was done by isopycnic centrifugation in CsCl-ethidium bromide followed by extraction with n-butanol, ethanol precipitation, phenol-chloroform extraction and chloroform extraction followed by an alcohol precipitation (Maniatis et al., 1982).

The same procedure was used to prepare M13 RFI DNA with the following modifications: JM103 was inoculated into YT medium and grown until a cell density of 1 x 10^8 cells per ml whereupon they were infected with M13 phage. After $2^1/_2$ to 3 hours they were treated as a plasmid preparation except that it was necessary to do two CsCl gradient centrifugations to minimize contamination of RFI with single-stranded DNA.

Small scale plasmid preparations (1.0 ml of bacterial culture) were obtained by the boiling method of Maniatis et al., (1982) except that prior to the ethanol precipitation step phenol-chloroform and chloroform extractions were done.

Small scale RFI M13 preparations were done similarly but like the large scale preparations JM103 was first grown to a cell density of 1 x 10^8 cells per ml, infected with M13 phage m.o.i. 20, and left to grow for $2^1/_2$ hours at 37°C. They were then treated as the boiling method for plasmid extraction described above.

2.07 Agarose gels

For analytical purposes the agarose gels used most often contained 0.8% agarose (Seakem) dissolved in running buffer (50 mM TrisHCl (pH 8.0), 5 mM Na acetate, 0.5 mM EDTA, 0.5 µg ethidium bromide/ml). To visualize the DNA, gels were photographed under short wave ultraviolet light (Maniatis et al., 1982).

2.08 Polyacrylamide Gels

For most purposes a 5% polyacrylamide gels cross linked with N,N'-methyl-bis-acrylamide (Bio-Rad) was run in 50 mM Tris-borate (pH 8.3), 1 mM EDTA buffer. After the gel had run sufficiently far (determined by the position of the

bromophenol blue dye), the gel was stained in 1µg/ml ethidium bromide for 10 min destained 5 min in H_2O and photographed under short wave ultraviolet light (Maniatis et al., 1982).

2.09 Isolation of specific DNA fragments

To isolate specific DNA fragments of greater than 700 bp in size by electroelution, agarose gels were made as above but with high purity agarose (Biorad). Progress of DNA fragment migration was monitored with a hand-held long wave ultraviolet light source. To elute the DNA, a trough was cut around the band of interest and a dialysis membrane fitted into the trough to collect the DNA when electrophoresis continued. The DNA sample taken from the trough was phenol-chloroform and chloroform extracted, ethanol precipitated and resuspended in TE buffer (Maniatis et al., 1982).

Fragments of less than 700 bp in size were eluted from 5% acrylamide gels by the 'crush and soak' method of Maxam and Gilbert (1980). The bands were visualized by staining with ethidium bromide (2.08). The DNA fragment was excised from the gel, cut into small pieces and added to 400 µl of elution solution (0.5 M CH_3COONH_4, 10 mM $(CH_3COO)_2Mg$, 1 mM EDTA, 0.1% SDS). This was left at 32°C in a rotating drum for 18 h; after a short spin the supernatant was saved, a

further 200 µl of elution sclution added and the mixture was left for 3 hours then collected. The two supernatant solutions were subsequently combined, extracted with phenol/chloroform and with chloroform and ethanol precipitated. This DNA could be used as it was or in cases where very clean DNA was needed, it was passed through an Elutip-D column.

2.10 In vitro DNA manipulation to produce blunt ends

To fill in recessed 3' ends, restricted duplex DNA was incubated in 50 mM TrisHCl (pH7.6), 10 mM $MgCl_2$ in the presence of the four deoxynucleoside triphosphates (at 0.5 mM) and Klenow fragment (1-5 U) for 15 min at 25°C (Maniatis et al., 1982).

Mung bean nuclease (Pharmacia) was used to remove 5' and 3' overhangs from restricted duplex DNA. The sample (50 µg/ml) was resuspended in buffer (30 mM Na acetate (pH 4.6), 50 mM NaCl, 0.5mM $ZnSO_4$, 5% glycerol), and 50 U of mung bean nuclease was added; the mix was incubated at 30°C for 20 min. The reaction was terminated by the addition of EDTA to 25 mM and extracted with phenol-chloroform and with chloroform. DNA was recovered by ethanol precipitation.

A second method employing S1 nuclease to remove 3' and 5' overhangs was used occasionally. One µg of restricted DNA was added to 50 µl of S1 nuclease buffer (30mM CH_3COONa (pH

4.5), 3 mM $ZnCl_2$, 0.3 mM NaCl) and 6 U of S1 and left at room temperature for 15 min. The reaction was halted by the addition of 20 mM EDTA followed by a phenol/chloroform and chloroform extraction and ethanol precipitation (Maniatis et al., 1982).

2.11 Dephosphorylation

Dephosphorylation of DNA fragments was done as described by Barker et al., (1983). The cleaved DNA was dissolved in 50 µl of 0.1 M TrisHCl (pH 8.4), and 2 U of Calf intestinal alkaline phosphatase was added and the reaction was left for 30 min at 55°C. The DNA was then extracted three times with phenol/chloroform and once with chloroform before being ethanol precipitated.

2.12 Phosphorylation of oligonucleotide inserts

An eight-base palindromic oligonucleotide (GCCATGGC) was phosphorylated by the method described by Zoller and Smith (1984). Four hundred pmoles of the oligonucleotide was suspended in kinase buffer (0.1 M TrisHCl (pH 8.2), 10mM $MgCl_2$ 10mM DTT), 1 mM ATP and 10 units of polynucleotide kinase were added in a volume of 20 µl. The reaction was left at 37°C for one hour and terminated by heating at 65°C for 10 min. The annealing of two such oligonucleotides

creates a double-stranded 8bp fragment which can be used in a ligation reaction with dephosphorylated vector.

2.13 Ligations

The reactions involved 100 ng of dephosphorylated vector with 10x molar amount of insert in ligation buffer (50 mM TrisHCl (pH 8), 10 mM $MgCl_2$, 20 mM DTT, 10 mM ATP) in a volume of 10 µl. Ten U of T4 DNA ligase was added to this and the reaction was left 18 h at 15°C (Maniatis et al., 1982). This ligated material was then used directly in subsequent transformations.

2.14 Transformations

These were done as described by Hanahan (1983) except that in general after heat shock the normal growth medium (detailed in section 2.03) for that strain of E. coli was added to the competent cells. In the case of the temperature-sensitive dnaA strains, better results were obtained by growing the bacteria in NY medium rather than SOB at 32°C. Selection for transformed cells was done in the first instance by selecting for the presence of an antibiotic gene carried by the introduced plasmid.

In the case of M13 transformation into JM103 the procedure was as above until the heat-shock step followed by

5 min on ice. Instead of being left at 37°C in media aliquots of the competent cells were added to molten top agar (0.7% agar, YT medium) along with 10 µl of 0.1M IPTG, 40 µl 2% X-gal, and some noncompetent JM103 to form a bacterial lawn for phage growth. The formation of plaques could be seen on the bacterial lawn and those phage which contained a subcloned fragment were identified by the absence of colour development upon disruption of the β-galactosidase gene.

2.15 Alkaline extraction

This is a method to confirm quickly the presence or absence of a plasmid in a clonal population. One ml of an overnight culture was centrifuged in a microfuge at 10,000 g. The pellet was resuspended in 80 µl of cracking buffer (50 mM Tris HCl (pH 7.6), 1% SDS, 2 mM EDTA, 0.4 mM sucrose, 0.1% bromophenol blue), mixed thoroughly with a toothpick, and left for 30 min at room temperature. The mix was then centrifuged in a Eppendorf tube for 15 min and the supernatant loaded onto an agarose gel (Maniatis et al., 1982).

2.16 Phage stocks

Lambda phage stocks were grown, stored and titered by the methods of Schlief and Wensink (1981). M13 phage

stocks were prepared as follows: YT medium was inoculated with JM103 and after a period of time sufficient for rapid cell division to be initiated M13 phage was added and left for approx. 18 h at 37°C. Then 1.5 ml of the culture was spun in a Eppendorf centrifuge for 5 min and the supernatant heat treated at 75°C for 10 min. This phage stock was in general 10^{11} p.f.u. per ml. When necessary M13 was titered by dilutions spotted on to a bacterial lawn of JM103 (Messing, 1983).

2.17 Complementation (C) Tests

This allows a quick characterization of M13 clones; 30 µl of the high titre phage stock is added to another clone's high titre phage stock which may have the same fragment insert but in another orientation. To the phage 3 µl of 0.5 M EDTA and 1.5 µl of 10% SDS is added to inhibit nucleases and to denature the phage coat. The mix is heated to 75°C for one hour and then left to anneal at room temperature for 1 h. Where the differing clones have complementary inserts the hybridized complex will run more slowly and can so be identified when the DNA is electrophoresed on an agarose gel (Messing, 1983).

2.18 Preparation of ssDNA for sequencing reactions.

Single-stranded DNA was prepared as reported by J. Messing (1983) but with the modifications of G. Mackie (personal communication).

JM103 cells were grown in YT medium until a cell density of .1 x 10^8 at which point they were infected (m.o.i of 20) with high titre M13 phage stock. The infection was allowed to proceed at 37°C for $5^1/_2$ to 7 h. Then 1.5 ml of the culture was centrifuged in a microfuge (10,000 g) for 5 min. The supernatent (1.2 ml) was removed and added to 300 µl of 25% PEG8000, 2.5 M NaCl. This was left at room temperature for 15 min then placed at 4°C for 5 min. Then the samples were centrifuged for 15 min in an microcentrifuge and the resulting precipitate resuspended in 800 µl of TE plus 0.5% sarcosyl, reprecipitated with 200 µl of the PEG solution as above and resuspended in 200 µl of TE. They were extracted with an equal volume of phenol equilibrated with 0.1 M Tris-HCl (pH 8), then with phenol/chloroform which had again been equilibrated with 0.1 M Tris-HCl (pH 8) and, lastly with 1:24 isoamyl alcohol:chloroform. The supernatant was adjusted to 2.5 M ammonium acetate and precipitated with ethanol. The pellet was resuspended in 45 µl of TE and 10 mM $MgCl_2$ and reprecipitated with 10 mM spermine and finally resuspended in 15 µl of TE at which point the DNA was used in the sequencing reactions.

2.19 DNA Sequencing

2.19a Dideoxy DNA Sequencing

Restriction fragments of the rep gene were subcloned into M13 derivatives. These M13-rep subclones were used as a source of ssDNA for sequencing using [α^{35}S]dATP and the dideoxy method Sanger et al., (1977) and Biggin et al., (1983). One µg of the single-stranded DNA prepared as described above was annealed to 2.5 pmol of 17-base primer, 1-2 U of DNA polymerase 1 Klenow fragment is added plus 10 µCi of [α^{35}S] dATP and the mixture split between 4 tubes. Tube one contained 0.166mM ddATP plus 0.125 mM of dCTP, dGTP, dTTP. Similarly the second tube contained 0.5 mM ddTTP, 8.33 µM dTTP and 0.17 mM of the nucleotides dCTP and dGTP. The third tube contained 0.1 mM ddCTP and 8.33 µM dCTP and 0.17 mM of dTTP and dGTP, lastly the fourth tube contained 0.1 mM ddGTP and 8.33 µM dGTP and 0.17 mM dCTP and dTTP. These tubes were incubated at 37°C for 20 min to allow complete incorporation of the radioactive dATP. A cold chase of dATP (50 µM) was added to complete any reactions prematurely terminated due to lack of dATP. These tubes were left a further 15 min and then the reaction was halted by the addition of formamide stop mix (95% deionized formamide, 10 mM EDTA, 0.5% bromophenol blue and 0.5% xylene cyanol).

In areas with substantial secondary structure either dITP was substituted for dGTP (Smith, 1979) and the quantity of ddGTP added in the fourth tube altered to 1 µM ddGTP to compensate for the lowered probability of Klenow using the dITP analogue. If this approach was not sufficient to produce a readable sequence, Sequenase (Tabor, 1987) or reverse transcriptase (Burton et al., 1981) were used instead of DNA polymerase I-Klenow fragment. In very severe cases it was sometimes necessary to switch to using the specific chemical degradation method of Maxam-Gilbert (1980).

2.19b Sequencing by Specific Chemical Degradation

When the Maxam-Gilbert specific chemical degradation method was used, the DNA substrate was usually 5'labeled with ^{32}P by T4 polynucleotide kinase (Barker et al., 1983). Ten µg of cleaved DNA was dephosporylated as described above and resuspended in 30 µl denaturation Buffer (10 mM TrisHCl (pH 9.5), 0.5 mM spermidine, 0.05 mM EDTA), heated at 70°C for 5 min. After transfer to 4°C the buffer was adjusted to 50 mM TrisHCl (pH 9.5), 10 mM $MgCl_2$, 5 mM DTT and 5% glycerol. Then 100 µCi of [gamma ^{32}P] dATP and 10 U of T4 polynucleotide kinase were added. After 30 min at 37°C 10 µl of 2M CH_3COONH_4 was added to halt the reaction, the volume adjusted to 100 µl and applied to a spun column (see

nick translation section). The DNA eluted from the spun column can then be used directly in a second cleavage reaction.

In cases where 5' labelling was not practical labelling was done at the 3' end with DNA polymerase I-Klenow fragment (Burton et al., 1981). Approximately 10 µg of each linearized plasmid DNA, was incubated in buffer (50 mM TrisHCl (pH7.6), 5 mM $MgCl_2$) containing dATP, dGTP and dTTP (at 0.8 mM each) and 50 µCi of [α^{32}P]dCTP (17 pmoles) in a volume of 100 µl. The mixture was incubated for 15 min at 23°C with 5 units of Klenow fragment, and the reaction terminated by phenol-chloroform extraction, followed by ethanol precipitation. The DNA pellet was resuspended in 20 µl of an appropriate restriction buffer and cut with a second restriction enzyme.

The end-labelled fragment of interest was purified from a 5% polyacrylamide gel by the 'crush and soak' method described previously. The DNA was further purified by elution through an Elutip-D column (Schleicher and Schuell) and ethanol precipitation. Equivalent amounts of radioactivity were added to Eppendorf tubes for each sequencing reaction (30,000 cpm each for C and G reactions, 60,000 cpm each for G+A and C+T reactions). Chemical modifications and cleavage reactions were performed as described by Maxam and Gilbert (1980).

2.20 Sequencing Gels

Samples were electrophoresed at 20 mA for 2 h on a 8% or 6% polyacrylamide gel which was cross-linked with N,N'-methylenebis-acrylamide (Bio-Rad) in 50 mM Tris-borate (pH 8.3)/ 1 mM EDTA, 7 M urea. The gel was then fixed in 10% acetic acid and dried onto Whatman 3MM paper before being exposed to XAR-5 film for autoradiography.

2.21 Analysis of Sequence

Sequence analysis was performed either with the programs of Larson and Messing, (1982) and Pongor et al., (1985) on an Apple II computer or with the programs of Stephens, (1985), Mount and Conrad, (1986), Langrimini et al., (1984) and the Protylze programs of Scientific and Educational Software Co. The latter programs were used on an IBM AT computer.

2.22 Isolation of total *E. coli* RNA

Cultures of *E. coli* MZ-1 cells carrying multicopy plasmid pRep0 (Colasanti and Denhardt, 1987) or *E. coli* RR1 carrying the pHBH30 (Bialkowska-Hobrzanska and Denhardt, 1984) plasmid were grown in L-broth with 100 µg/ml carbenicillin to a density of $2x10^8$ cells/ml. The MZ-1 cells

were grown at 30°C and the P_L promoter was induced by incubation at 42°C for 45 min. The RR1 cells were grown at 37°C.

Nucleic acids from these cells were isolated by the SDS/boiling method of Dennis and Nomura (1975) and the RNA purified by the guanidinium/cesium chloride procedure (Glisin et al., 1974). The sample was dissolved in 5 ml of buffer (4 M guanidinium isothiocyanate, 5 mM citrate (pH 7.0), 0.1 M ß-mercaptoethanol, 0.5% Sarkosyl) and centrifuged in a CsCl gradient (1 g of CsCl per 2.5 ml suspension) to pellet the RNA into a 5.7 M CsCl cushion. The RNA was dissolved in TE with 1% SDS, extracted with chloroform/butanol (4:1), ethanol precipitated and resuspended in distilled water.

2.23 Mapping the site of rep mRNA transcription initiation

Previous work in our lab had suggested that the rep promoter was located between the restriction sites NruI of position 1 on the DNA sequence (Fig 3) and EcoRI position 457 (Bialkowska-Hobrzanska et al., 1985).

The DNA was 5' end-labelled at the EcoRI restriction site as described in section 2.19b; approximately 10 ng of purified uniquely labelled fragment with a specific activity of 2 x 10_5 cpm/µg was added to 150 µg of total cell RNA prepared from cells carrying the pHBH30 plasmid. This was

suspended in 30 µl of 80% formamide, 0.4 M NaCl, 40 mM PIPES (piperazine-N,N'-bis(2 ethanesulfonic acid) (pH6.5) 1mM EDTA. After 15 min at 80°C the nucleic acids were incubated for 18 h at 37°C . The hybridization reactions were then adjusted to 400 µl with chilled S1 nuclease buffer (0.03 M sodium acetate (pH4.5), 0.25 M NaCl, 1 mM $ZnSO_4$, 5% glycerol and 20 µg/ml denatured calf thymus DNA) containing 40 U of S1 nuclease (Calbiochem). Samples were incubated for 60 min at 37°C then ethanol precipitated using 5 µg of carrier tRNA (Weaver and Weissmann, 1979). The DNA was then resuspended in sequencing stop mix (see 2.19a).

2.24 S1 mapping of the 3' end of rep mRNA transcripts.

The 3'-terminal portion of rep is located on a Sau3AI-HindII fragment extending between positions 2324 and 2592. The slightly larger 310-bp Sau3AI fragment was isolated, 3' end labelled as above, cut with HindII and the resulting 268-bp Sau3AI-HindII fragment isolated and hybridized to RNA from E. coli MZ-1 transformed with pRep0, a plasmid expressing the rep gene at high levels (Colasanti and Denhardt, 1987). The S1 nuclease digestion was performed as described above.

2.25 Western blots to visualize the Rep protein

To compare the *in vivo* levels of the Rep protein in the TC and *rep* strains, and in strains where Rep was produced by *rep*-containing plasmids in a *rep*⁻ host, cultures of these *E. coli* were grown in L-broth at 30°C (temperature-sensitive strains) or 37°C to a cell density of 1x10⁸ cells/ml. Ten ml of these cells were spun down and resuspended in 0.5 ml of 15% sucrose, 50 mM Tris(HCl) (pH 8), 10 mM EDTA and subjected to a 3-sec sonication at 7 kilocycles per sec after which they were spun for 5 min in an Eppendorf centrifuge to remove cell debris. The quantity of protein was then determined by absorbance at 280nm and equivalent amounts of protein were added to an equal quantity of 2x Laemmli sample buffer (160 mM TrisHCl, (pH 6.8), 4% SDS, 20% glycerol, 1.4 M β-mercaptoethanol, 0.04% bromophenol blue) and boiled for 5 min. These samples were then loaded on a SDS-polyacrylamide gel (10% acrylamide, 1% bisacryamide) with a 4.5% polyacrylamide stacking gel which was then run until the bromophenol blue dye reached the bottom of the gel. This separated the Rep protein from others on the basis of size. Immunoblotting was performed essentially as described by Towbin *et al.* (1979) with the following modifications. After electrophoresis, proteins were transferred from the gel onto nitrocellulose paper (0.45 μm, BA85, Schleicher and Schuell) in electro-

blotting buffer (1 litre contains 3 g of Trizma base (Sigma), 14.4 g of glycine and 200 ml methanol) using a BioRad Trans-Blot Cell. After transfer (0.1 A for 18 h), the portion of the nitrocellulose sheet on to which the protein markers had been blotted was stained with Amido Black dye (0.1% Amido Black, 50% methanol, 10% acetic acid) for 10 min, washed 3 times for 5 min each in distilled water and put aside. The remainder of the blot was washed 3 times for 10 min in Tris-buffered saline (TBS: 20 mM TrisHCl (pH 7.5), 150 mM NaCl) and 6 times for 5 min in TBS containing 0.05% Tween 20 (Batteiger et al., 1982) and then the blot was incubated for at least an hour in a solution of TBS/0.05% Tween 20 and 4% BSA to block non-specific adsorption. Rabbit antiserum (provided by S. Matson) raised against the Rep protein was diluted 1/750 in TBS/0.05% Tween 20 4% BSA and incubated with the blot in a volume of 5 ml for 2 h at room temperature. The blots were washed for 6 or 7 times, 10 min each, in TBS/0.05% Tween 20 followed by a wash with TBS/0.05% Tween /4% BSA for an hour and an incubation with a biotinylated goat anti-rabbit second antibody. After washing 6 times with TBS/Tween, the blot was reacted with avidin-horse radish peroxidase and washed 3x with TBS/Tween and 3x PBS (Swack et al., 1987). The presence of Rep protein was determined by reacting the peroxidase with substrate (0.5 mg/ml diaminobenzidine, 0.03% $NiCl_2.6H_2O$, 0.02% H_2O_2 in PBS)

2.26 Nick translation of DNA

Nick translation as described by Rigby (1977) was used to create ^{32}P-labelled DNA as probe in plaque hybridization, Southern blot analysis and slot blot analysis. The DNA fragment (100 ng) to be used as a probe was suspended in buffer (50 mM TrisHCl (pH 7.6), 5mM $MgCl_2$) containing all four deoxynucleoside triphosphates (dNTP); usually 2, 3 or 4 [α-^{32}P]dNTP's were used (1 µl of 50 µCi/ml, 2.5 µM) supplemented with the complementary unlabelled dNTP's (1 mM). This mixture was incubated with DNase I (Worthington) at 2.5 µg/ml for 3 min at room temperature and then E. coli DNA polymerase I (1-2 U) was added and incubation continued for 2 h at 15°C. The reaction was terminated with 25 mM EDTA, and unincorporated dNTP's were separated from labeled DNA using the spun column technique as described in Maniatis (1980). The plunger of a 1 ml syringe was removed, the bottom plugged with sterile glass wool, and coarse Sephadex G-50 (Pharmacia) (prepared as manufacturers directions) was added and subjected to a 2.6k spin; this was repeated until the bed volume of the spun column reached 0.9 ml. The column was then washed 3x with 100 µl applications of TE buffer and the sample applied in 100 µl and the mixture was spun as above and the eluate collected.

2.27 Colony Hybridization

This was done identically for both M13 and lambda plaques (Maniatis et al., 1982). The phage were plated out in agarose top agar to reduce background and after an overnight incubation at 37°C they were then put at 4°C for 1 h. Then a nitrocellulose paper circle was placed for 2-3 min on the surface of these plates. The nitrocellulose was then placed for 5 min on 3MM paper soaked in 0.5 M NaOH, and then transferred to 3MM paper soaked in 0.1 M NaOH, 1.5 M NaCl for 20 sec. It was then twice left for 20 sec on 3MM paper soaked with 0.5 M TrisHCl, 1.5 M NaCl. The nitrocellulose blot was air dried for 30 min, baked at 80°C for 1 hour, placed in a Kapak bag, and pre-hybridized in hybridization solution (50% formamide, 6X SSC, 10X Denhardt's solution, 100 µg/ml salmon sperm DNA) for at least 4 h at 42°C. Approximately $1x10^6$ cpm of nick-translated probe (boiled for 5 min) was added to each filter in hybridization solution and the incubation continued for 16-20 h at 42°C. Filters were then washed for 15 min each time for 3 times in 2X SSC, 0.1% SDS and once in 0.2X SSC, 0.1% SDS, dried and exposed to Kodak XAR-5 film with an intensifying screen at -70°C for autoradiography.

2.28 Southern Blot Analysis

Analysis of DNA by hybridization with nick-translated probes was accomplished by transferring the DNA from an agarose gel to a nitrocellulose sheet (0.45 µm, BA-85, Schleicher and Schuell). The DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min and then neutralized by soaking in 0.5 M TrisHCl (pH 7.5), 1.5 M NaCl for 30 min. DNA was transferred to the nitrocellulose by the method described by Southern (1975) in 20X SSC buffer overnight. After transfer, the filters were baked in vacuo at 80°C for 2-3 h and treated as the colony blots.

2.29 Construction of a dasC lambda lysogen

The phage lambda repk1 was constructed as detailed by Colasanti and Denhardt (1987). It involved subcloning a 6-kb fragment from 83.5 min on the E. coli chromosome into lambda and replacing the rep gene it contained with a marker kanamycin gene. J. Colasanti then isolated rare lysogens of E. coli dasC strains TC862 and TC861.

2.30 Small scale lambda preparation

This is a modification of the procedure of Helms et al., (1985). Lambda phage was prepared as for a high titre

lambda phage stock (2.15), diluted by one volume of 10 mM Tris HCl (pH 7.5) and applied to a DEAE-cellulose column which had been washed with 10 mM TrisHCl (pH 8), 10 mM $(CH_3COO)_2Mg$, 60 mM CH_3COONa). The lambda was eluted by 10 mM TrisHCl (pH 8), 50 mM $(CH_3COO)_2Mg$. To the eluate $^1/_{10}$ volume of TES was added (100 mM TrisHCl (pH 8), 100 mM EDTA, 1% SDS) and 10 µg of proteinase K. This mixture was incubated at 37°C for 1 h, extracted with phenol-chloroform, chloroform and ethanol precipitated.

2.31 Large scale lambda preparation

The E. coli Y1090 cells were grown with good aeration until the cell density reached 2 x 10^8 cells per ml in LB medium. The medium was adjusted to 10 mM $MgSO_4$, infected with high titre lambda phage stock (m.o.i. 1.0) and left for a further 3-4 hours until the cells lysed; $^1/_{400}$ th the volume of chloroform was added and the culture was left shaking for 10 more min, then centrifuged at 5,000 rpm for 15 min. The supernatant was then treated with 0.8 µg/ml ribonuclease A and 0.8 µg/ml DNaseI and left at 4°C for 1 hour. The solution was then adjusted to 4.6% NaCl and 8% of polyethylene glycol-8000 and left on ice for 4 hours. This was centrifuged for 15 min at 5,000 rpm and the pellet saved. The pellet was resuspended in $^1/_{100}$ the volume of the original culture with CsCl (density 1.5 g/ml) in SM

buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM TrisHCl(pH 7.5) and 0.01% gelatin) centrifuged for 20 hours in a SW50.1 rotor at 3500 rpm, and the viral band collected. Three vol of H_2O and 8 vol of ethanol were added and the phage left to pellet for 15 hours at -20°C. The phage were then centrifuged at 7500 rpm for 15 min, and the phage pellet was dried, resuspended in 5 mM TrisHCl (pH 8), and incubated for 1 h at 37°C with 500 µg/ml of proteinase K. The DNA was then extracted with phenol-chloroform, chloroform and precipitated by the addition of $^1/_4$ volume of CH_3COONH_4 and two volumes of ethanol.

2.32 Isolation of the dasC mutation

Several lysogens with lambda repK integrated into the rep locus were cured and the lambda colonies screened with an internal fragment of the rep gene which had been deleted in lambda repK1 construction. The DNA from the clones which gave a positive signal was isolated and restricted with XhoI. The phage isolates that yielded a 6 kb fragment characteristic of lambda carrying the dasCrep gene were further analyzed by the transfer of the 6 kb DNA to nitrocellulose by the method of Southern detailed in section 2.28. These blots were probed with a ^{32}P-labelled internal fragment of rep (EcoRI(457)-HindIII(1120). The dasC DNA was then isolated and subcloned into pBR322, pSP64 or pSP65. The

new plasmids were tested for their ability to supress the ts dnaA46 phenotype when supplied in trans to TC182.

2.33 Total chromosomal DNA from E. coli

DNA was prepared by a modification of the method described by Davis et al., (1980). Small aliquots of bacterial cultures (0.5 to 1.0 ml) were pelleted and resuspended in 0.5 ml of lysis buffer (50 mM TrisHCl (pH 8.3), 50 mM EDTA, 15% sucrose, 1 mg/ml lysozyme and left at room temperature for 10 min. SDS was added (10 µl) and the preparations incubated at 70°C for 5 min. After heating, potassium acetate (50 µl of 5.0 M) was added and the tubes left on ice for 1 h. The potassium-dodecyl sulfate-protein complex was pelleted by centrifugation (12000 g/15 min) and 1 ml of absolute ethanol added to the supernatant to precipitate the nucleic acids. The precipitate was re-suspended in 200 µl of TE extracted once with phenol-chloroform (phenol was distilled and equilibrated with TE, (pH 8.0) and precipitated with 1/3 vol of 10 M CH_3COONH_4, 2 vol absolute ethanol. The pellet was washed with 70% ethanol, dissolved in a small volume of TE, and quantified by A260 measurement.

2.34 Determination of the growing fork number in *E. coli*

The surK▲*rep* and TC strains were grown in NY medium until the culture reached a density of $1x10^8$ cells per ml. Total bacterial nucleic acid was isolated by the method described above. Equivalent nucleic acid samples of various dilutions were transferred to nitrocellulose filters in duplicate with an S & S Mini-fold II slot blotting apparatus. One of the duplicate blots was hybridized with a nick-translated DNA probe containing the *oriC* region of *E. coli*; the second blot was hybridized with a probe derived from the region of the chromosome where DNA replication terminates. After autoradiography, the individual slots were cut from the nitrocellulose sheet and the bound radioactivity quantified by scintillation counting. To prepare the probe DNA, the origin fragment (5 kb) was isolated from pSY317 (Yasuda and Hirota, 1977) by *Eco*RI cleavage; the terminal region was derived from pJH113 (Hensen and Kuempel, 1985) as a 5 kb *Bam*HI/*Hin*dIII fragment. Each fragment, purified from a gel by electroelution, was nick-translated, and approximately $1x10^6$ cpm of ^{32}P-labeled, denatured probe DNA was hybridized to each blot in the same manner as DNA blots.

CHAPTER 3

SEQUENCE OF THE rep GENE AND PROTEIN

3.01 ABSTRACT

The sequence of a 2.67-kilobase section of the Escherichia coli chromosome that contains the rep gene has been determined. This gene codes for a protein of predicted Mr 72,800, a DNA helicase, which is also a ssDNA-dependent ATPase. The sequenced region contains a transcribed open reading frame of the correct length and orientation to encode the rep protein. A secondary structure for the protein can be formulated from the amino acid sequence. We have compared both the primary and the secondary structures of rep with other proteins and find the greatest homology between rep and E. coli helicase II, the product of the uvrD gene.

* Some of the results in this chapter have been published in Bialkowska-Hobrzanska, H., Gilchrist, C.A. and Denhardt, D.T.(1985) J. Bact. 164, 1004-1010. and Gilchrist, C.A. and Denhardt, D.T. (1987) Nuc. Acids.Res. 15 465-475.

3.01 INTRODUCTION

The rep gene of Escherichia coli codes for the Rep helicase, a ssDNA-dependent ATPase which maps to 83.5 min on the E. coli genetic map between the ilvC and rho genes.

The DNA from this region of the E. coli genome has been subcloned by Clark and Carbon (1976) into a plasmid designated pLC-7 which when introduced into rep⁻ E. coli strains restores rep function (Scott and Kornberg, 1978). The loss and restoration of the Rep helicase was ascertained by studying the propagation of the ΦX174 phage. Due to the fact that the Rep helicase is required for the replication of the ΦX174 and other isometric and filamentous single-stranded DNA phages, these phage will not grow on rep⁻ strains unless the lesion in the rep gene has been complemented in trans.

While the Rep helicase is not essential for cell viability, the replication forks in rep⁻ strains of E. coli move at about half their average rate and there is an apparently compensatory increase in the number of replication forks (Lane and Denhardt, 1975; Colasanti and Denhardt, 1987).

The complementation of rep⁻ with smaller fragments of E. coli DNA isolated from the pLC-7 plasmid led to the conclusion that the rep gene resided in a 2.67-kb fragment which extended from a NruI to a BalI restriction site.

Major deletions at the internal EcoRI or HindIII or HpaI sites abolished Rep activity (Bialkowska-Hobrzanska and Denhardt, 1984).

In order to discover the location of possible promoters in this area and, if present, their direction of transcription, a fragment extending from the internal EcoRI site mentioned above to past the NruI site and so outside the designated rep area to an upstream XhoI site was subcloned 5' to the structural portion of a ß-galactosidase gene (the promoter, Shine and Dalgarno sequence, and the first 4 amino acid codons had been deleted). The fragment extending from the internal HpaI site mentioned above to a downstream PstI restriction site was similarly subcloned. The use of the PstI site meant that this fragment also extended past the minimal area required for rep complementation. Transcription of this ß-galactosidase gene only occurred if a promoter resided in the cloned fragment.

Transcription of the rep/ß-galactosidase gene constructions was followed by probing, with a ^{32}P-labeled ß-galactosidase fragment, total cell RNA from lac strains which carried the plasmid constructs. The results indicated that the rep promoter transcribed through the EcoRI restriction site towards the BalI site and hence resided in the NruI-EcoRI fragment (Bialkowska-Hobrzanska et al., 1985).

3.03 RESULTS AND DISCUSSION

3.04 S1 mapping of the site of transcription initiation

The EcoRI restriction site at nucleotide 452 in Fig 3 was 5' labelled with T4 polynucleotide kinase. The resulting EcoRI-NruI restriction fragment was hybridized to 150µg of total cell RNA isolated from a strain carrying the rep gene on a multicopy plasmid and hence having an elevated level of rep mRNA.

The unhybridized single-stranded nucleic acid was digested by S1 nuclease and the protected DNA was run out on an 8% sequencing gel alongside the results of specific chemical cleavage of the same fragment (done by the method of Maxam and Gilbert). After taking into consideration the fact that this method of sequencing leads to the destruction of one nucleotide, I determined that the site of transcription initiation was at nucleotide residue 388-a C nucleotide (Fig 1). This is unusual in that it is more usual for transcription initiation to occur with either an A or G nucleotide. The region at around 388 is a C-rich region; this is reminiscent of the start site of stringently controlled promoters (Travers, 1980). In bacterial promoters subjected to stringent control there appears to be a heptanucleotide sequence spanning the transcription start site CCCCNCC which

Fig 1 **S1 mapping of the 5' end of Rep mRNA**

The RNA was hybridized to a ^{32}P-5' end-labeled restriction fragment, subjected to S1 digestion and electrophoresed on a 8% polyacrylamide sequencing gel as described in Materials and Methods (2.23). Since one nucleotide is destroyed in the reaction, these data indicate that the major start site is at base 388.

is similar to that appearing at the rep transcription initiation site CCTCCCC. There have been no experiments done so far to investigate whether rep is controlled by this system.

3.05 Nucleotide sequence

The functional rep gene is contained within a 2.67-kb region of E. coli DNA defined by NruI and BalI restriction sites (Bialkowska-Hobrzanska et al., 1985). This fragment maps between ilvC and rho at about 83.5 min (Bialkowaska-Hobrzanska and Denhardt, 1984). The strategy used to sequence it is shown in Fig 2. All regions were sequenced in both directions. Fig 3 presents the nucleotide sequence of this DNA and the amino acid sequence of the Rep protein.

There is no region in the NruI - EcoRI fragment which is identical to the consensus promoter sequence of Hawley and McClure (1983); however this is not unexpected as the Rep protein is present as only 20 copies per cell and it is not necessary for the promoter to be very efficient. There are a number of promoter-like sequences present in the sequence but the S1 mapping evidence restricts the possibilities to the sequence close to nucleotide residue 388. The residues 378 - 384 -TACAAT come close to the consensus for the -10 promoter region (Hawley and McClure, 1983); 17 bp upstream (Aoyama et al., 1983) is the sequence GTCACA

Fig 2. **The sequencing strategy for rep**

The region shown is the section of E. coli DNA sequenced; the hatched box covers the rep gene. The line below is a restriction enzyme map of rep showing only sites used to construct M13rep subclones, restriction endonucleases are designated respectively Nr, NruI; A, AsuII; RI, EcoRI; B, BssHII; Bs, BstEII; RV, EcoRV; H2, HindII; Av, AvaII; H3, HindIII; Hp, HpaI; N, NcoI; S, Sau3AI; M, MluI; C, ClaI; R, RsaI; Mn, MnlI; Ba, BalI.

The continuous arrows indicate the direction of the dideoxy sequencing; their length is proportional to the amount of sequence determined from an individual clone. Dotted lines indicate sequence determined by the specific chemical degradation method.

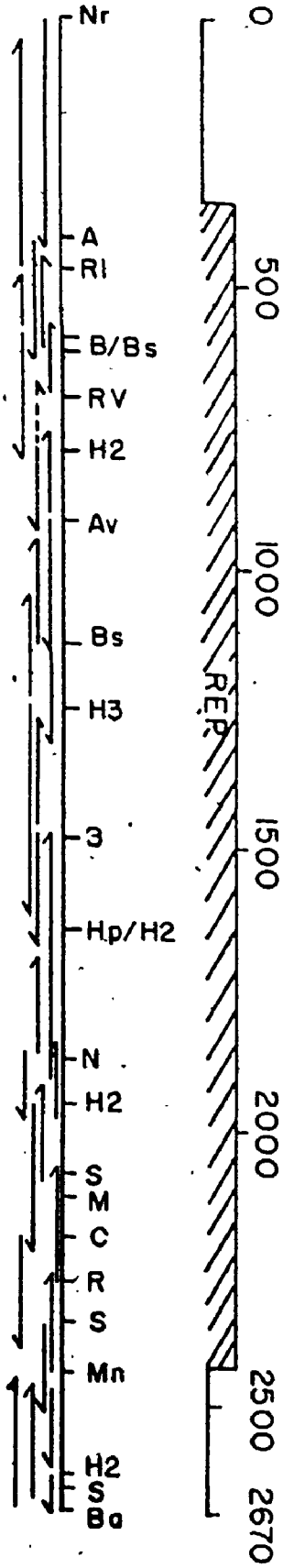
Nr
A
RI
B/Bs
RV
H2
Av
Bs
H3
3
Hp/H2
N
H2
S
M
C
R
S
Mn
H2
S
Ba
REP.
0
500
1000
1500
2000
2500
2670

Fig. 3. **The sequence of the rep gene and protein**

The sequence numbering begins at the NruI restriction site and extends to the BalI restriction site. The -35 and -10 sequences of the promoter are enclosed in boxes, the Shine-Dalgarno sequence is underlined twice, and the site of initiation of rep mRNA transcription is marked with an arrow. The ATG initiation codon is at nucleotide 419. The rep mRNA terminates at nucleotide 2360, which is overlined and marked with a T. The amino acid residues resembling the 'A' consensus sequence (Walker et al., 1982) described in the text and in Table 4, are underlined.

```
                                                                    58
gagtggttgataatggaacgacgatgcatgcgttgccattaattttccgttgag             56

cacgacaactaacctgtttttatgatttcaggaggatgcggccatcgtaatacg             111

tcgtgctgaacggtccattttggctaacaggataggttaccaaagttgcattcca            166

gtatttcaccaaccttagggctgaaattaatcgccacgagtaaacctctccttag            221

taaactctgaaaaagtaataacacaacgttacgacccgatattttctaagtctaa            276

tggattcacgatgaactccgatttcggtcttctttctctgatttaaacatccgca            331

gccaaccggttagcggcttacacgcg[gtcaca]ttcaaatgcgattctgc[tac]           383
-10     mRNA                    -35
[aat]cctccccccgttcgaagattgagcaatacacct  ATG CGT CTA AAC             430
                                        Met Arg Leu Asn

CCC GGC CAA CAA CAA GCT GTC GAA TTC GTT ACC GGC CCC TGC             472
Pro Gly Gln Gln Gln Ala Val Glu Phe Val Thr Gly Pro Cys

CTG GTG CTG GCG GGC GCG GGT TCC GGT AAA ACT CGT GTT ATC             514
Leu Val Leu Ala Gly Ala Gly Ser Gly Lys Thr Arg Val Ile

ACC AAT AAA ATC GCC CAT CTG ATC CGC GGT TGC GGT TAT CAG             556
Thr Asn Lys Ile Ala His Leu Ile Arg Gly Cys Gly Tyr Gln

GCG CGG CAC ATT GCG GCG GTG ACC TTT ACT AAT AAA GCA GCG             598
Ala Arg His Ile Ala Ala Val Thr Phe Thr Asn Lys Ala Ala

CGC GAG ATG AAA GAG CGT GTA GGG CAG ACG CTG GGG CGC AAA             640
Arg Glu MET Lys Glu Arg Val Gly Gln Thr Leu Gly Arg Lys

GAG GAG CGT GGG CTG ATG ATC TCC ACT TTC CAT ACG TTG GGG             682
Glu Glu Arg Gly Leu MET Ile Ser Thr Phe His Thr Leu Gly

CTG GAT ATC ATC AAA CGC GAG TAT GCG GCG CTT GGG ATG AAA             724
Leu Asp Ile Ile Lys Arg Glu Tyr Ala Ala Leu Gly MET Lys

GCG AAC TTC TCG TTG TTT GAC GAT ACC GAT CAG CTT GCT TTG             766
Ala Asn Phe Ser Leu Phe Asp Asp Thr Asp Gln Leu Ala Leu

CTT AAA GAG TTG ACC GAG GGG CTG ATT GAA GAT GAC AAA GTT             808

Leu Lys Glu Leu Thr Glu Gly Leu Ile Glu Asp Asp Lys Val

CTC CTG CAA CAA CTG ATT TCG ACC ATC TCT AAC TGG AAG AAT             850
Leu Leu Gln Gln Leu Ile Ser Thr Ile Ser Asn Trp Lys Asn

GAT CTC AAA ACA CCG TCC CAG GCG GCA GCA AGT GCG ATT GGC             892
Asp Leu Lys Thr Pro Ser Gln Ala Ala Ala Ser Ala Ile Gly
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GAG CGG GAC CGT ATT TTG CTC CAT TGT TAT GGG CTG TAT GAT  934
Glu Arg Asp Arg Ile Leu Leu His Cys Tyr Gly Leu Tyr Asp

GCA CAC CTG AAA GCC TGT AAC GTT CTC GAC TTC GAT GAT CTG  976
Ala His Leu Lys Ala Cys Asn Val Leu Asp Phe Asp Asp Leu

ATT TTA TTG CCG ACG TTG CTG CTG CAA CGC AAT GAA GAA GTC 1018
Ile Leu Leu Pro Thr Leu Leu Leu Gln Arg Asn Glu Glu Val

CGC AAC GGC TGG CAG AAC AAA ATT CGC TAT CTG CTG GTG GAT 1060
Arg Asn Gly Trp Gln Asn Lys Ile Arg Tyr Leu Leu Val Asp

GAG TAT CAG GAC ACC AAC ACC AGC CAG TAT GAG CTG GTG AAA 1102
Glu Tyr Gln Asp Thr Asn Thr Ser Gln Tyr Glu Leu Val Lys

CTG CTG GTG GGC AGC CGC GCG CGC TTT ACC GTG GTG GGT GAC 1144
Leu Leu Val Gly Ser Arg Ala Arg Phe Thr Val Val Gly Asp

GAT GAC CAG TCG ATC TAC TCC TGG CGC GGT GCA CGT CCG CAA 1186
Asp Asp Gln Ser Ile Tyr Ser Trp Arg Gly Ala Arg Pro Gln

AAC CTG GTG CTG CTG AGT CAG GAT TTT CCG GCG CTG AAG GTG 1228
Asn Leu Val Leu Leu Ser Gln Asp Phe Pro Ala Leu Lys Val

ATT AAG CTT GAG CAG AAC TAT CGC TCT TCC GGG CGT ATT CTG 1270
Ile Lys Leu Glu Gln Asn Tyr Arg Ser Ser Gly Arg Ile Leu

AAA GCG GCG AAC ATC CTG ATC GCC AAT AAC CCG CAC GTC TTT 1312
Lys Ala Ala Asn Ile Leu Ile Ala Asn Asn Pro His Val Phe

GAA AAG CGT CTG TTC TCC GAA CTG GGT TAT GGC GCG GAG CTA 1354
Glu Lys Arg Leu Phe Ser Glu Leu Gly Tyr Gly Ala Glu Leu

AAA GTA TTA AGC GCG AAT AAC GAA GAA CAT GAG GCT GAG CGC 1396
Lys Val Leu Ser Ala Asn Asn Glu Glu His Glu Ala Glu Arg

GTT ACT GGC GAG CTG ATC GCC CAT CAC TTC GTC AAT AAA ACG 1438
Val Thr Gly Glu Leu Ile Ala His His Phe Val Asn Lys Thr

CAG TAC AAA GAT TAC GCC ATT CTT TAT CGC GGT AAC CAT CAG 1480
Gln Tyr Lys Asp Tyr Ala Ile Leu Tyr Arg Gly Asn His Gln

TCG CGG GTG TTT GAA AAA TTC CTG ATG CAA AAC CGC ATC CCG 1522
Ser Arg Val Phe Glu Lys Phe Leu MET Gln Asn Arg Ile Pro

TAC AAA ATA TCT GGT GGT ACG TCG TTT TTC TCT CGT CCT GAA 1564
Tyr Lys Ile Ser Gly Gly Thr Ser Phe Phe Ser Arg Pro Glu

ATC AAG GAC TTG CTG GCT TAT CTG CGC GTG CTG ACT AAC CCG 1606
Ile Lys Asp Leu Leu Ala Tyr Leu Arg Val Leu Thr Asn Pro
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GAC GAT GAC AGC GCA TTT CTG CGT ATC GTT AAC ACG CCG AAG 1648
Asp Asp Asp Ser Ala Phe Leu Arg Ile Val Asn Thr Pro Lys

CGA GAG ATT GGC CCG GCT ACG CTG AAA AAG CTG GGT GAG TGG 1690
Arg Glu Ile Gly Pro Ala Thr Leu Lys Lys Leu Gly Glu Trp

GCG ATG ACG CGC AAT AAA AGC ATG TTT ACC GCC AGC TTT GAT 1732
Ala MET Thr Arg Asn Lys Ser MET Phe Thr Ala Ser Phe Asp

ATG GGC CTG AGT CAG ACG CTT AGC GGA CGT GGT TAT GAA GCA 1774
MET Gly Leu Ser Gln Thr Leu Ser Gly Arg Gly Tyr Glu Ala

TTG ACC CGG TTC ACT CAC TGG TTG GCA GAA ATC CAG CGT CTG 1816
Leu Thr Arg Phe Thr His Trp Leu Ala Glu Ile Gln Arg Leu

GCG GAG CGG GAG CCG ATT GCC GCG GTG CGT GAT CTG ATC CAT 1858
Ala Glu Arg Glu Pro Ile Ala Ala Val Arg Asp Leu Ile His

GGC ATG GAT TAT GAA TCC TGG CTG TAC GAA ACA TCG CCC AGC 1900
Gly MET Asp Tyr Glu Ser Trp Leu Tyr Glu Thr Ser Pro Ser

CCG AAA GCC GCC GAA ATG CGC ATG AAG AAC GTC AAC CAA CTG 1942
Pro Lys Ala Ala Glu MET Arg MET Lys Asn Val Asn Gln Leu

TTT AGC TGG ATG ACG GAG ATG CTG GAA GGC AGT GAA CTG GAT 1984
Phe Ser Trp MET Thr Glu MET Leu Glu Gly Ser Glu Leu Asp

GAG CCG ATG ACG CTC ACC CAG GTG GTG ACG CGC TTT ACT TTG 2026
Glu Pro MET Thr Leu Thr Gln Val Val Thr Arg Phe Thr Leu

CGC GAC ATG ATG GAG CGT GGT GAG AGT GAA GAA GAG CTG GAT 2068
Arg Asp MET MET Glu Arg Gly Glu Ser Glu Glu Glu Leu Asp

CAG GTG CAA CTG ATG ACT CTC CAC GCG TCG AAA GGG CTG GAG 2110
Gln Val Gln Leu MET Thr Leu His Ala Ser Lys Gly Leu Glu

TTT CCT TAT GTC TAC ATG GTC GGT ATG GAA GAA GGG TTT TTG 2152
Phe Pro Tyr Val Tyr MET Val Gly MET Glu Glu Gly Phe Leu

CCG CAC CAG AGC AGC ATC GAT GAA GAT AAT ATC GAT GAG GAG 2194
Pro His Gln Ser Ser Ile Asp Glu Asp Asn Ile Asp Glu Glu

CGC CGG CTG GCC TAT GTC GGC ATT ACC CGC GCC CAG AAG GAA 2236
Arg Arg Leu Ala Tyr Val Gly Ile Thr Arg Ala Gln Lys Glu

TTG ACC TTT ACG CTG TGT AAA GAA CGC CGT CAG TAC GGC AAC 2278
Leu Thr Phe Thr Leu Cys Lys Glu Arg Arg Gln Tyr Gly Asn

TGG TGC GCC CGG AGC CGA GCC GCT TTT TGC TGG AGC TGC CGC 2320
Trp Cys Ala Arg Ser Arg Ala Ala Phe Cys Trp Ser Cys Arg
```

T

AGG ATG ATC TGGtttgggaacaggagcgcaaagtggtcagcgccgaagaacg 2372
Arg MET Ile TER

gatgcagaaaggcaaagcatctggcgaatctgaaagcgatgatggcggcaaaacg 2427

agggaaataatcaaggccggaaaagatgcgtcagcatcgcatccggcacttactc 2482

attaatgcacttccagcggccagtggacatagctctgccactgctttcctgagca 2537

ataatctctttacccagcggatgttgggttagccaaccttgcggaaacgtcaagg 2592

tcaacagttcatggttagcctgtaatgtcatctctggcacgagatcgtcacggcg 2647

acggctggccaacaatgatggcca 2670

Table 3

AMINO ACID COMPOSITION %

48 hour hydrolysis with 0.01% 2- mercaptoethanol

(cys. met. trp. are not included)

amino acid	1st Exp.	2nd Exp.	3rd Exp.	pred
asp	11.4	9.8	11.1	10.5
thr	7.1	8.7	6.7	6.5
ser	7.4	7.4	9.7	6.3
glu	12.3	6.3	13.6	14
pro	2.5	3.4	2.3	3.4
gly	8.4	8.5	11.4	6.8
ala	8.5	9.8	8.1	8.4
val	5.6	5.9	4.9	5.4
ile	6.0	6.1	2.0	5.6
leu	13.2	17.2	7.4	13.5
tyr	-	-	2.7	3.86
phe	4.0	5.0	2.9	4.4
his	3.7	3.0	4.5	2.6
lys	9.5	8.5	7.0	5.8
arg	0.4	0.4	5.7	8.25

which although not as close to the consensus as the -10 is could act as a satisfactory -35 region (the consensus for -35 is TTGACA Hawley and McClure, 1983). The sequence GAGCAA at 405-411, probably acts as the Shine-Dalgarno ribosome binding site, it like the -35 sequence, is futher than normal from the consensus. As mentioned earlier, however, the low quantity of Rep helicase present in the E. coli cell suggests that high rates of transcription and translation do not occur.

The translational start AUG codon is at nucleotide 419. The open reading frame predicts a protein of Mr 72,802 that terminates at position 2329. The relative molecular weight of the rep protein estimated on SDS-polyacrylamide gels has been variously reported around 67,000 to 70,000 by different laboratories (Tessman and Peterson, 1982; Scott and Kornberg, 1978; Takahashi et al., 1978). The sequence of the amino terminus of the Rep protein was determined by T.G. Flynn as Met-Arg-Leu-Asn-Pro which is the predicted N-terminus sequence of the open reading frame. Three amino acid determinations of Rep by W. Chung are shown in Table 3.

3.06 The 3' end of rep mRNA

The S1 mapping of the 3' end of the rep gene shown in Fig. 4 reveals that the mRNA either terminates at, or is subject to, an RNA processing event at DNA in region 2360.

Fig. 4 **S1 mapping of the 3' end of rep mRNA**

The RNA isolated from MZ-1 carrying the pRep0 plasmid (2.22 materials and methods) was hybridized to Sau3AI-HindII fragment labeled at the 3'end with [α^{32}P]dCTP, subjected to S1 digestion, and electrophoresed on an 8% polyacrylamide sequencing gel alongside Sau3AI-cut pBR322 (materials and methods 2.24). The positions of the three marker fragments in this region are indicated.


31
36
46

The region upstream of nucleotide 2360 does not have the characteristic structural features of a classical Rho-independent terminator - i.e. a region of dyad symmetry followed by a run of T's (Christie et al., [illegible]4). In fact the region following the translational stop codon of rep, 2329-2360, appears to lack significant secondary structure; this lack of secondary structure agrees with current theory regarding the requirements for Rho-dependent termination (Bear et al., 1985). However, with Rho-dependent terminators there is generally an untranslated region of at least 78 nucleotides. In contrast, the noncoding 3' part of the rep mRNA extends for only 30 nucleotides and, moreover, has a low C content (18%); Rho seems to bind more strongly to mRNA having a high C content (Bear et al., 1985). These facts do not rule out the possibility that mRNA ends at 2360 due to a transcriptional termination event since other terminators also lack secondary structure and long untranslated 3' regions (Cone et al., 1983).

3.07 'A' ATPase consensus sequence

The Rep protein possesses a region with good homology in its N-terminal region to the "A" consensus ATPase sequence of Walker et al. (1982). This sequence, G-X_4-GKT-X_6-I; Table 4, located at amino acid residues 22-36 in rep, corresponds to the "A" sequence, which is present in most

Table 4

Comparison of the 'A' ATPase consensus sequence found in Rep and UvrD

Protein	Residues	Sequence
'A' Sequence		[G] ---- [GKT] ------ [I]
Rep	19-40	LVL [A] GAGS [GKT] RVITNK [I] AHLI
		*** * **** *** ** * * *
UvrD	25-46	LVL [A] GAGS [GKT] RVLVHR [I] AWLM

The amino acids boxed are those of the A consensus sequence Walker et al (1982);

* indicates identical amino acids in both Rep and UvrD

ATP-utilizing enzymes so far sequenced. The other ATPase consensus sequence, identified by Walker, the "B" sequence, is not present in rep. It is of interest that the "A" consensus sequence is located in a region of ß-pleated sheet structure. It seems to be a common feature of this sequence that, where secondary structures have been predicted (Husain et al., 1986; Nakayama et al., 1984), it is located in or near a ß-pleated sheet. The protein sequence close to the "A" site is unusual in that while being hydrophobic it has a positive charge; this may indicate that it is involved in binding to DNA or to ATP since these have a negative charge. However "A" has not yet been shown to be present in the ATP binding site itself (Knight and McEntee, 1985).

In order to clarify the importance of this sequence, rep mutants were created which either have this region removed or have an insertion downstream in the positive hydrophobic area. The first mutation, 3-1, consists of a deletion between the internal EcoRI site (452) and the N-terminal SstII site (538). In order to retain the correct open reading frame after the 5' overhangs generated by these two enzymes were removed by the Mung Bean nuclease, the vector was dephosphorylated, and a 8-bp fragment (CGGTACCG) (see 2.12) was inserted (Fig 5). This deletion of 30 aa removed an entire strand of the ß-pleated sheet (Fig 6).

The Rep3-1 mutant protein allele was then supplied in trans to a rep⁻ host and the origin/terminus ratio deter-

Fig 5. **'A' ATPase mutants of rep**

Panel 1 shows a direct comparison of the DNA sequence of wild type rep, the deletion mutant rep3-1 and the insertion mutant rep8-6. The wild type allele has been run in lanes A,C,G,T; rep3-1 in lanes A',C',G',T'; and rep8-6 in lanes A",C",G",T". The SstII(538) restriction site indicates the point where the sequences diverge. Panel 2, the sequence of rep3-1 . Panel 3, rep8-6 . Panel 4, wild type rep. The KpnI oligonucleotide is denoted by a line alongside.

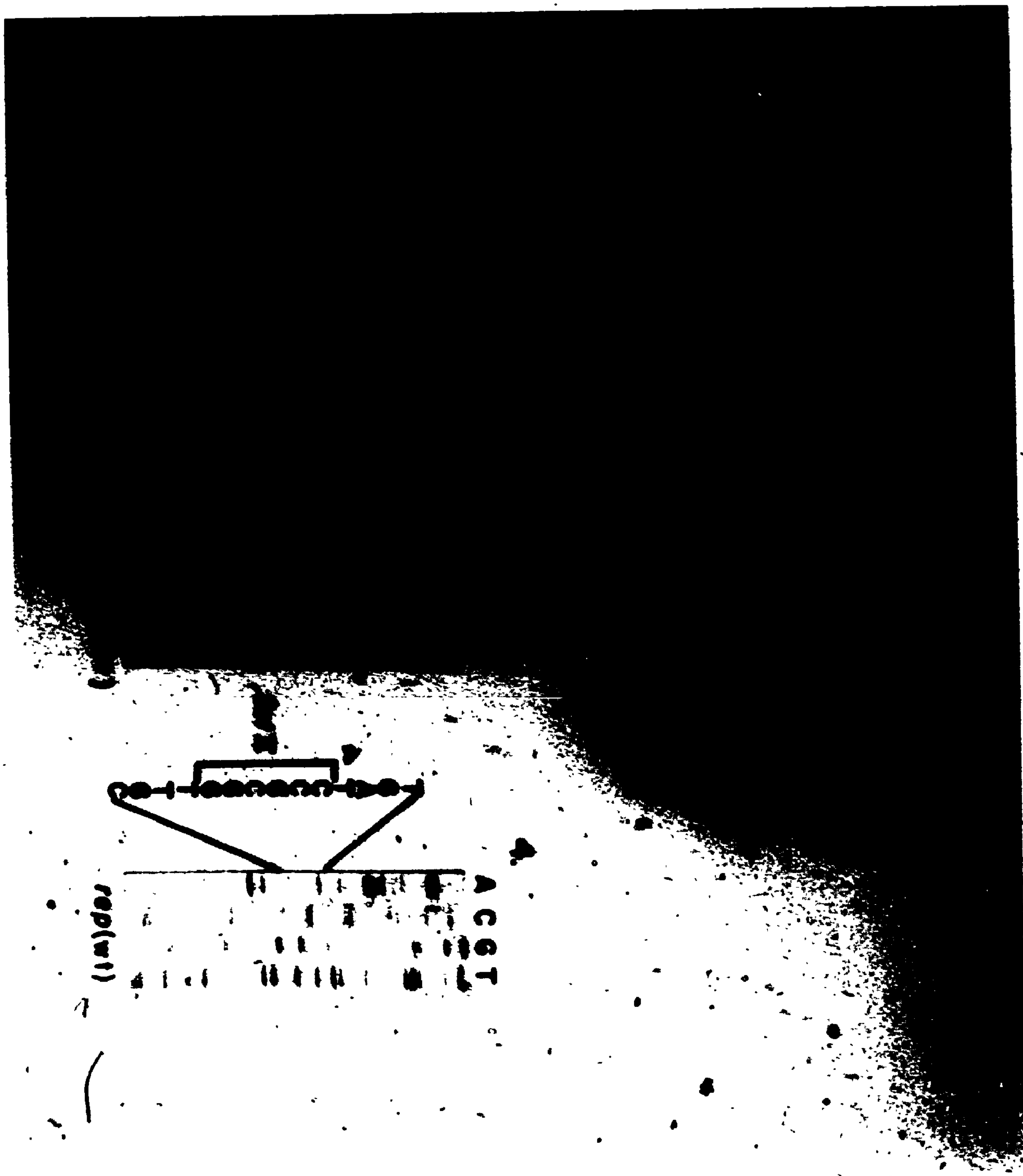

A C G T
rep(wt)

Fig 6. **Analysis of the alteration of Rep in 'A' ATPase mutants**

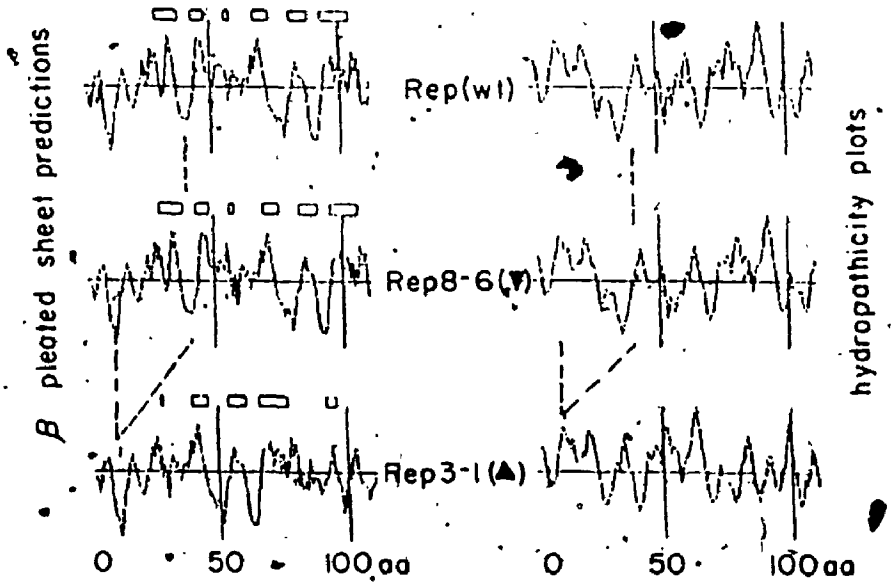
The top section compares rep and Rep wild type with rep3-1, Rep3-1, and rep8-6 and Rep8-6. Bases derived from the KpnI linker are underlined the restriction sites EcoRI(452) and SstII(538) are overlined. In the amino acid sequence the 'A' consensus amino acid residues are in heavy type. The lower section compares the ß-pleated sheet predictions of Rep, Rep3-1 and Rep8-6 and the hydropathicity profiles of Rep, Rep3-1 and Rep8-6 at site of the mutation.

Alignment of wild type and 3-1 (deletion) alleles

```
          EcoRI(452)
CAAGCTGTCGAATTCGTTACCGGCCCCTGCCTGGTGCTGGCGGGCGCGGGTTCCGGTAAAA rep(wt)
::::::::::                                                :::::
CAAGCTGTCG----------------------------------------------CGGTA--- rep3-1(▲)

                                        SstII(538)
CTCGTGTTATCACCAATAAAATCGCCCATCTGATCCGCGGTTGCGGTTATCAGGCGCGGC  rep(wt)
                                       ::: ::::::::::::::::::::::
----------------------------------------CCG-GGTTGCGGTTATCAGGCGCGGC  rep3-1(▲)

1       EcoRI                                  SstII
MRLNPGQQQAVEFVTGPCLVLAGAGSGKTRVITNKIAHLIRGCGYQARHIAAVTFTNKAA Rep(wt)
::::::::::                             :    ::::::::::::::::::
MRLNPGQQQAV-------------------------AVP--GCGYQARHIAAVTFTNKAA Rep3-1(▲)
```

Alignment of wild type and 8-6 (insertion) alleles

```
EcoRI(452)
GAATTCGTTACCGGCCCCTGCCTGGTGCTGGCGGGCGCGGGTTCCGGTAAAACTCGTGT rep(wt)
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GAATTCGTTACCGGCCCCTGCCTGGTGCTGGCGGGCGCGGGTTCCGGTAAAACTCGTGT rep8-6(▼)

                                SstII(538)
TATCACCAATAAAATCGCCCATCTGATCCGC------GGTTGCGGTTATCAGGCGCGGC rep(wt)
::::::::::::::::::::::::::::<        ::::::::::::::::::::::
TATCACCAATAAAATCGCCCATCTGATCCCGGTACCGGGTTGCGGTTATCAGGCGCGGC rep8-6(▼)

1       EcoRI                                  SstII
MRLNPGQQQAVEFVTGPCLVLAGAGSGKTRVITNKIAHLIR--GCGYQARHIAAVTFTNK Rep(wt)
::::::::::::::::::::::::::::::::::::::::   ::::::::::::::::::
MRLNPGQQQAVEFVTGPCLVLAGAGSGKTRVITNKIAHLIPVPGCGYQARHIAAVTFTNK Rep8-6(▼)
```



mined. The origin/terminus ratio is increased in the absence of Rep but can be returned to wild type levels when the Rep protein is supplied in trans. Rep3-1 is defective in this function (Table 5).

This indicates an increased probability that the 'A' sequence is significant in Rep function. However the deletion could cause perturbation of Rep3-1 protein structure. As mentioned above the deletion of these 30 aa removes a section of the ß-sheet and there may be other changes not indicated in either the secondary structure predictions (Fasman and Fasman, 1978) or hydropathicity profiles (Doolittle and Doolittle, 1982) generated by the "Protylze" programs. These are shown in Fig 6. These graphs do not show any new features caused by the new juxtaposition of the sequences which had previously bordered the deletion.

A second mutation 4 aa downstream of the 'A' region, was constructed. The rep gene was cleaved with SstII and the 5' overhang treated mildly with Mung Bean Nuclease, the DNA was then dephosphorylated and ligated to the 8 bp fragment described above. The resulting mutant, Rep8-6 does not appear to be altered in secondary structure (Fig. 6). The measurement of this mutant's capacity to complement rep- mutants as judged by its affect on the origin/terminus ratio indicates that while it is not completely ineffective it is not functioning at normal rates (Table 5).

Table 5

Comparison of origin/terminus ratios in surK(▲*rep*) strains supplied with differing *rep* alleles

surK/plasmid	Origin/Terminus
pSP64	8 ± 0.5
pSP*rep*(N-P)	4 ± 0.5
pSP*rep*3-1	7.5± 0.5
pSP*rep*8-6	6 ± 0.5

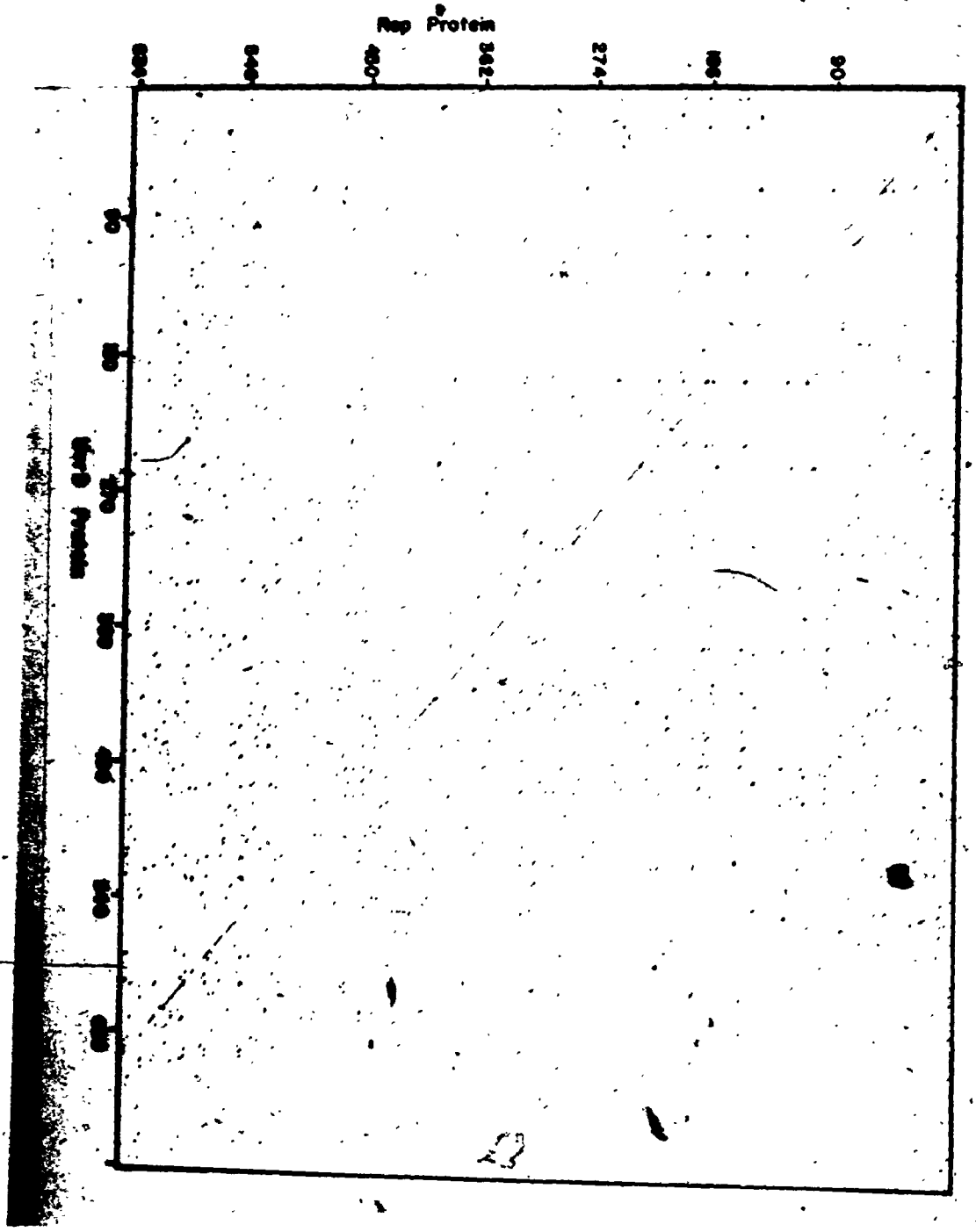
3.08 Homology with uvrD

A search through the 1985 National Biomedical Research Foundation protein data bank and GenBank revealed that there was homology between rep and a portion of the uvrD gene. This prompted us to perform the dot matrix analysis shown in Figure 7 using the sequence of uvrD reported by Finch and Emmerson (1984) and the sequence we have deduced for the Rep protein. As can be seen, there is impressive homology throughout the two sequences, approximately 40%, raising the possibility that the two proteins have evolved from a common progenitor. Both helicases are ssDNA-dependent ATPases that, after binding to DNA, proceed along it in a 3' to 5' direction (Yarranton and Gefter, 1979; Matson, 1986). Both proteins possess a region with good homology to the "A" consensus ATPase sequence of Walker et al., (1982) in their N-terminal region (Table 4). The other ATPase consensus sequence, identified by Walker, the "B" sequence, is not present in either rep or uvrD. There is homology between rep and uvrD surrounding the site of the "A" sequence.

Despite these similarities there are differences in how these proteins function. For example, rep can initiate unwinding at a nick in the DNA, whereas uvrD requires a 12-bp gap. A study of the sequences that have diverged in these two proteins, and the synthesis of chimeric proteins, may reveal how this modulation of function is effected.

Fig. 7 **Homology between the Rep and UvrD proteins.**

The comparison was made using the dot matrix program of Stephens (1985). A dot was generated if any two amino acids in a window of three matched. The Rep protein sequence is on the vertical axis, UvrD on the horizontal axis.


Rep Protein
450
362
274
186
90

3.09 Codon usage

Table 6 compares the use of codons in rep with uvrD and with an average of "non-regulatory" genes (Konigsberg and Godson, 1983) in E. coli. In E. coli the codons used in highly expressed genes are generally those that correspond to abundant tRNA species, presumably in order to facilitate rapid translation of the mRNA (Gouy and Gauthier, 1982; Konigsberg and Godson, 1983). This will not regulate the quantity of protein expressed in itself (Sharp and Li, 1986) but is in general symptomatic for the rate of gene expression. Highly expressed genes include those coding for the ribosomal proteins and uvrD, whose product is present at 5000-8000 copies per cell (Abdel-Monem and Hoffmann-Berling, 1980). However, uvrD is an anomalous E. coli gene in that it uses more rare codons than usual. These codons are also utilized to a greater extent in rep, but in contrast to DNA Helicase II, rep is present at a level of only 50 copies per cell (Scott and Kornberg, 1978). The codon usage in the rep gene resembles that found in dnaG, (Konigsberg and Godson, 1983) whose product is expressed at low levels. In rep, the GAG (Glu), CGG (Arg), and GGG (Gly) rare codons are used 2-5 times more frequently than average. The CCA (Pro) codon is not used at all although its average use in E. coli proteins is approximately 20%.

Table 6

Codon Usage in Rep compared to uvrD and an "average" E. coli protein[a].

Amino Acid	Codon	rep Codons	% SYNONYM USE rep	uvrD	E.coli
Phe	UUU	16	64	45.5	44
	UUC	9	36	54.5	56
Leu	UUA[i]	2	2.5	8	6.1
	UUG[i]	13	17	10	8
	CUU[i]	7	9	8	9
	CUC[i]	6	8	15.5	7
	CUA[i]	2	2.5	2.5	2
	CUG	47	61	68	69
Ile	AUU	12	38	48	37
	AUC	19	59	52	62
	AUA[i]	1	3	0	1
Met	AUG	20	-	-	-
Val	GUU	6	19	12	38
	GUC	8	26	12	13
	GUA	2	6.5	16.5	23
	GUG	15	48.5	59.5	27
Ser	UCU	4	11	6	27
	UCC	7	19.5	9	26
	UCA[i]	0	0	3	8
	UCG[i]	7	19.5	26	11
	AGU[i]	5	14	9	6
	AGC	13	36	47	22
Pro	CCU[i]	2	11	5	9
	CCC[i]	3	17	5	6
	*CCA	0	0	20	20
	CCG	13	72	70	65
Thr	ACU	8	21.5	9	24
	ACC	14	38	47	51
	ACA[i]	2	5.5	6	6
	ACG[i]	13	35	38	20
Ala	GCU	6	12.5	8	28
	GCC	13	27	36	19
Tyr	UAU	15	68	26	41
	UAC	7	32	74	59
His	CAU	7	47	61	39
	CAC	8	53	39	61
Gln	CAA[i]	10	33	11	27
	CAG	20	67	89	73
Asn	AAU[i]	9	32	33	24
	AAC	19	68	67	76
Lys	AAA	24	73	67	77
	AAG[i]	9	27	33	23
Asp	GAU	21	66	35	51
	GAC	11	34	65	49
Glu	GAA	24	48	59	73
	*GAG[i]	26	52	41	27
Cys	UGU	3	37.5	50	42
	UGC	5	62.5	50	58
Trp	UGG	9	-	-	-
Arg	CGU	15	32	38	58
	CGC	22	47	47.5	35
	CGA[i]	2	4	3	2
	*CGG[i]	7	15	11.5	3
	AGA[i]	0	0	0	1
	AGG[i]	1	2	0	0.2
Gly	GGU	14	36	33	48
	GGC	4			

a Codon frequencies are those obtained from 25 "nonregulatory" genes (Konigsberg and Godson, 1983)

* An unusual codon use in rep.

i Infrequently used codon in E. coli (Konigsberg and Godson, 1983)

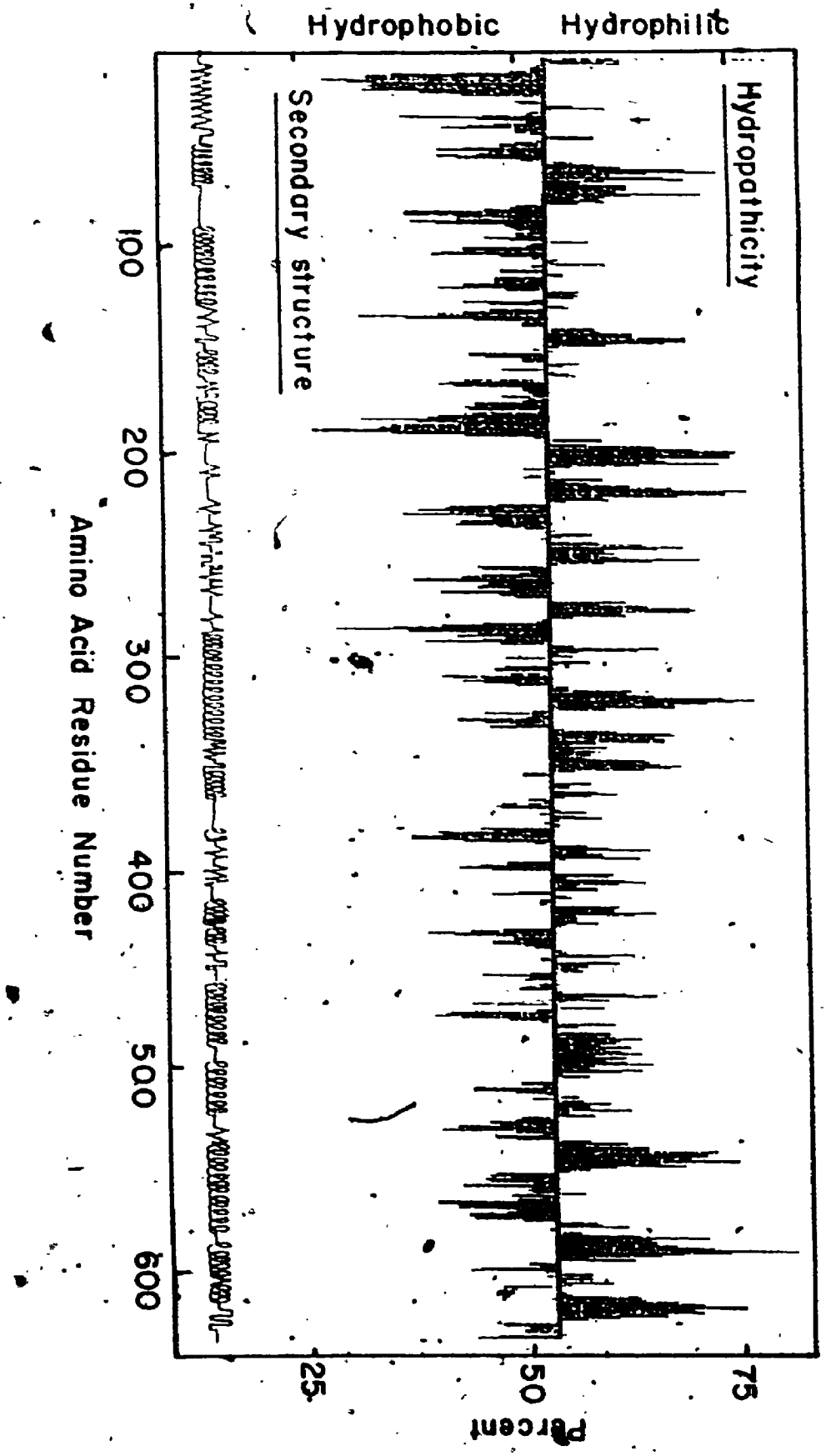
3.10 Secondary Structure

Although the calculations available for predicting secondary structure are only approximate, I have deduced a possible secondary structure for rep assuming that the amino acid sequence of the protein is the major determinant of its folding. I used the program of Pongor et al., (1985) and the equations of Garnier et al., (1978) to generate a secondary structure of rep, which is shown in Figure 8 along with a hydropathicity plot for rep (Stephens, 1985). The formulae predict rep's α-helical content as approximately 46% and β-pleated sheet content to be 21%.

As mentioned above the "A" consensus sequence is located in a region of β-pleated sheet structure in most ATP binding proteins and this is true of Rep's also. As Rep is known to bind to double-stranded DNA at a nick in the DNA I looked for the DNA-binding domain helix-turn-helix (Pabo and Sauer, 1984). I did not detect such a domain in Rep. A comparison between the secondary structures of UvrD and Rep shows regions of similarity that are more extensive than the primary sequence homology referred to above requires and therefore may be related to function.

Fig. 8 **The hydropathicity profile of Rep and its calculated secondary structure**

The plot indicates the degree of hydrophobicity of rep, with 100%-0% being equivalent to the solvent parameter values 3 to -3.4: each bar on the graph is calculated from ten residues. The line diagram indicates the ability of rep to form secondary structure: ℓℓ is α-helix, ∧ β-pleated sheet, ∫ turns, — · random coil. The arrow indicates the 'A' ATPase site.


Hydrophobic
Hydrophilic
Hydropathicity
Secondary structure
100
200
300
400
500
600
Amino Acid Residue Number
25
50
75
Percent

CHAPTER 4

ANALYSIS OF THE dasCrep ALLELE

4.01 ABSTRACT

The dasC suppressor locus of the dnaA gene maps at about 83.5 min in the E. coli chromosome. The rep gene which encodes a DNA helicase also maps to this region.

I mapped the dasC locus more precisely by isolating a NruI/BalI fragment of 2.67 kb. This was capable of conferring the temperature resistance phenotype to dnaA46 mutant strains when supplied in trans. As this DNA encodes the rep gene we investigated Rep in these strains by comparing wild type in vivo levels of Rep and the effect of Rep on initiation at the origin. The primary sequence of rep was looked at as well as the ability of the dasCrep allele to suppress temperature sensitive dnaA strains other than dnaA46.

It was found that the lesion responsible for the dasC phenotype was in the region of DNA downstream of rep which appears to regulate Rep/DnaA interactions.

4.02 INTRODUCTION

The DnaA protein is essential to E. coli and acts to permit initiation of E. coli DNA replication at oriC. The initial step in initiation appears to be the binding of this protein to the oriC R sequence TTAT(C/A)CA(C/A)A (Zyskind and Smith, 1986).

In order to locate and study proteins which may interact with the dnaA protein, Atlung et al., (1981) isolated and then analyzed spontaneously occurring mutations outside the dnaA gene that reversed the temperature-sensitive dnaA46 mutation.

One of the suppressor loci, designated dasC, was mapped by use of transducing lambda ilv phages and located between the ilvC and rho genes (Fig 9). The location of the dasC suppressor mutation meant that it was either in or close to the rep gene which I had earlier sequenced. The rep gene product is a DNA helicase which is also a ssDNA-dependent ATPase. The Rep protein had not been previously identified as having a role in initiation though a possible connection was the fact that in rep mutants there is a change in the timing of initiation that is thought to compensate for the decrease in the rate of elongation at the replication forks (Lane and Denhardt, 1975; Colasanti and Denhardt, 1987).

The rep gene is not an essential protein for E. coli growth. In reconstituted in vitro systems rep is not

required for the initiation of DNA replication and the essential gene product DnaB acts as a helicase during initiation of DNA replication (Baker et al., 1986). However the loss of the Rep helicase can be lethal to the cell in cases where the rep mutation is combined with either a mutation in ssb (Tessman and Peterson, 1982), uvrD (Taucher-Scholz et al., 1983) or certain rho mutants (Fassler et al., 1985).

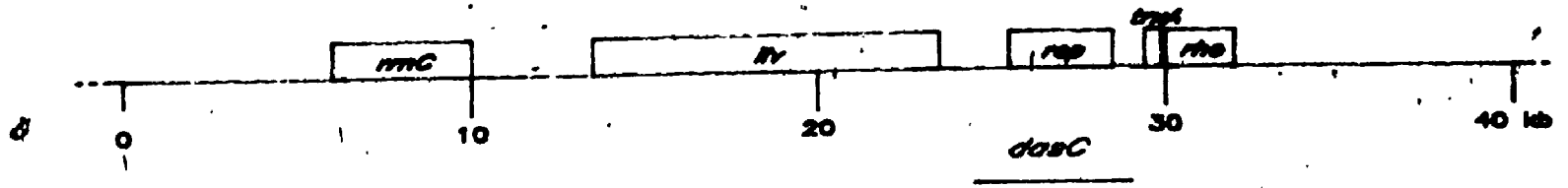
In order to clarify the role of rep in E. coli DNA replication initiation, I examined Rep protein levels in vivo in the mutant strains TC182 (temperature sensitive due to the dnaA46 mutation), TC861 (carrying the dnaA46 mutant allele but capable of growth at 42°C due to the presence of the dasC mutation), TC862 (a second dasC strain) and compared this information with that obtained from the original wild type TC152 strain.

The DNA capable of conferring the dasC phenotype was isolated and its ability to complement other dnaA and rep mutations when supplied in trans was examined. The dasC phenotype in the TC861 and TC862 strains was due to a mutation outside the complementing rep DNA. This mutation resided within a region that appears to antagonize a possible the Rep/DnaA interaction.

Fig 9. **Map of the E. coli. chromosome at 83.5 min.**

The information relating to the position of dasC was obtained from T. Atlung (1981) the position of trxA from (Lim et al 1985)

The table scores growth at the restrictive and permissive temperatures of the TC strains.



Strain	32 °C	42 °C
TC152 *rep*$^+$ *dnaA*$^+$	+	+
TC182 *rep*$^+$ *dnaA46* (ts)	+	−
TC861 sup *dasC* *dnaA46* (ts)	+	+
TC862 sup *dasC* *dnaA46* (ts)	+	+

4.03 **RESULTS**

4.04 *In vivo* analysis of the *dasC* and wt *rep* alleles

A comparison was made between the levels of Rep protein expressed in TC152 cells, where the *rep* and the *dnaA* genes are wild type, and the mutant strains TC182, which has a wild type *rep* gene but has the temperature sensitive *dnaA46* allele, and TC861 and TC862, which also contain the *dnaA46* mutant allele but are now capable of growth at 42°C due to the presence of a second mutation, *dasC*.

These *E. coli* strains were grown up at 32°C, until they reached a concentration of 1 x 10^8 cells/ml, pelleted, resuspended in a sucrose Tris/EDTA buffer and subjected to sonication. The cellular debris was removed and the quantity of protein present in each sample was determined by its adsorbance at 280 nm. Laemmli loading buffer was added to equivalent quantities of the supernatant. This was then run out on a 10% acrylamide gel; blotted onto nitrocellulose (see materials and methods 2.25) and the resulting "Western" blot exposed first to anti-*rep* antibody, secondly to biotinylated goat anti-rabbit antibody, then finally to versin/horse radish peroxidase. The location of Rep on the nitrocellulose blot was identified by the reaction of the peroxidase with substrate. The blot shows no change in Rep

Fig 10 **Quantity of Rep in TC strains.**

The figure shows a western blot of proteins from the TC strains which has been probed with an anti-Rep antibody. In this figure the Mr of protein standards are indicated on the right side. Lane a is purified Rep protein; b, protein isolated from TC152 (wt) (although this signal appears elevated this is not the case in repetition of this experiment); c, protein isolated from TC182 dnaA46 (ts), d protein isolated from TC861 (dasC), e, protein isolated from TC862 (dasC); f, protein isolated from surK(▲rep) (despite the fact that this is a rep▲ strain some signal is observed due to the cross reaction of the antibody) ; g, protein isolated from cells carrying rep (wt) on a multicopy plasmid.

a
b
c
d
e
f
g
-92,500
-66,200
-45,000

protein levels (the 70kd band) in the different strains TC182 (*dnaA46* ts), TC861 (das) and TC862 (das) (Fig. 10).

In vivo studies of Rep function consisted of testing for the growth of the ΦX174 phage after infection. Since Rep is essential for the replication of this phage, the fact that the ΦX174 phage are capable of growing in all TC strains tested indicates that according to this assay Rep is functioning normally with respect to ΦX growth in cells expressing the *dasC* phenotype.

The origin/terminus ratios in the TC strains were determined by the method described in materials and methods, section 2.34. The DNA was isolated as in section 2.33 and then probed with either the origin or terminus ^{32}P-labelled DNA fragments. The quantity of probe hybridized was compared by quantifying the signal strength on XAR-5 film.

It had been found previously that the origin/terminus ratio of DNA isolated from *rep*$^-$ strains is altered (Lane and Denhardt, 1975). This was thought to be due to a decrease in the rate of progression of the replication forks, which leads to a compensatory change in the timing of initiation.

The origin/terminus ratios in the supressor *dasC* TC861 and TC862 strains were compared with that of the temperature sensitive TC182 or wild type TC152 and the control surK$^-$*rep* strain and surK$^-$*rep*/pSP*rep*(N-P) plasmid where the Rep protein is supplied in trans. Very little difference was found among the TC strains, other than those accountable by

Fig 11. **Origin/terminus ratio of TC strains.**

The upper panel graphs the origin/terminus ratio of the TC strains at 32°C, 37°C, 40°C, 42°C and for comparison the values of surK(▲rep) and surK/pSPrep(N-P). The data was obtained from densitometary scan of the slot blots in the lower panel which show the quantity of origin or terminus ^{32}P-labelled probe hybridising to 1 µg (origin probe) or 2.5 µg (terminus probe) of genomic DNA isolated from the TC strains at the indicated temperatures. (the DNA was applied in three differing dilutions (not shown) in order to be certain the result was quantitative).

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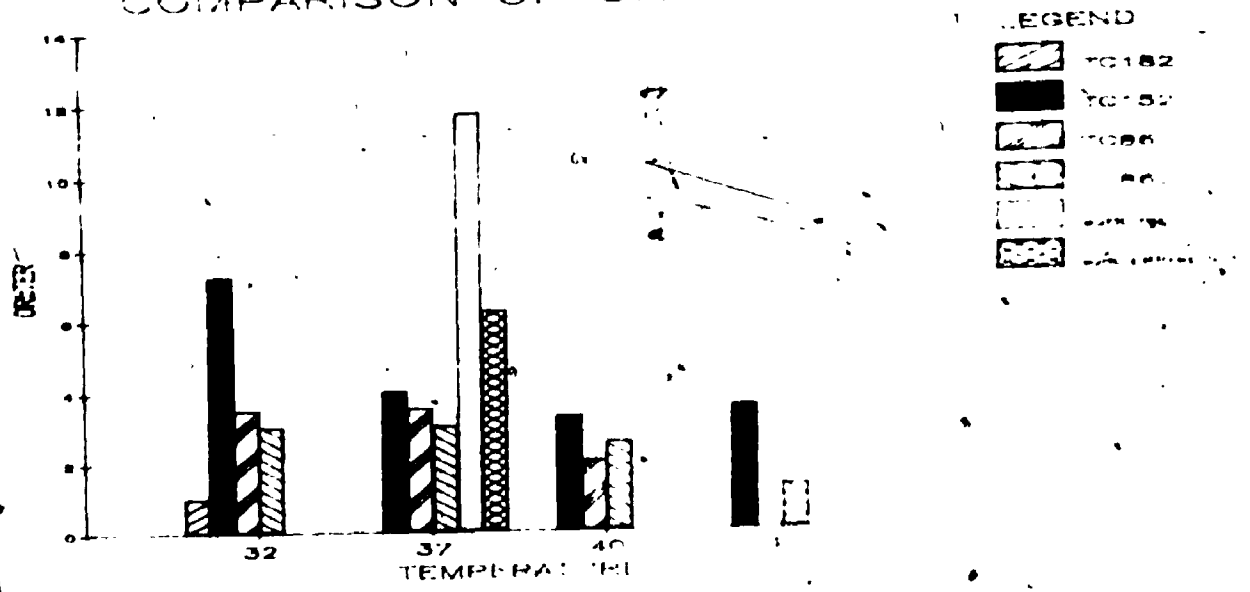
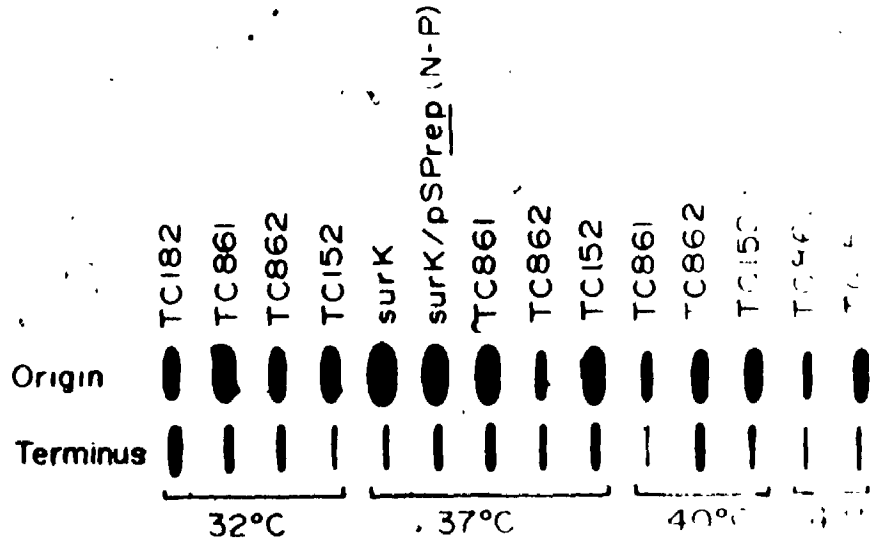
COMPARISON OF ORI TO TER RATIOS





differences between the dasC, dnaA46 and wild type strains growth rates at the selected temperatures (Fig 11). In contrast the ratio in the surK▲rep cells and the surK /pSPrep(N-P) cells (Fig 11) different - reflecting the loss of the Rep as found by Colasanti and Denhardt, (1987).

The results indicate that as judged by these criteria, Rep was not altered in this aspect of its helicase activity.

4.05 Isolation of the rep gene from the dasC strains

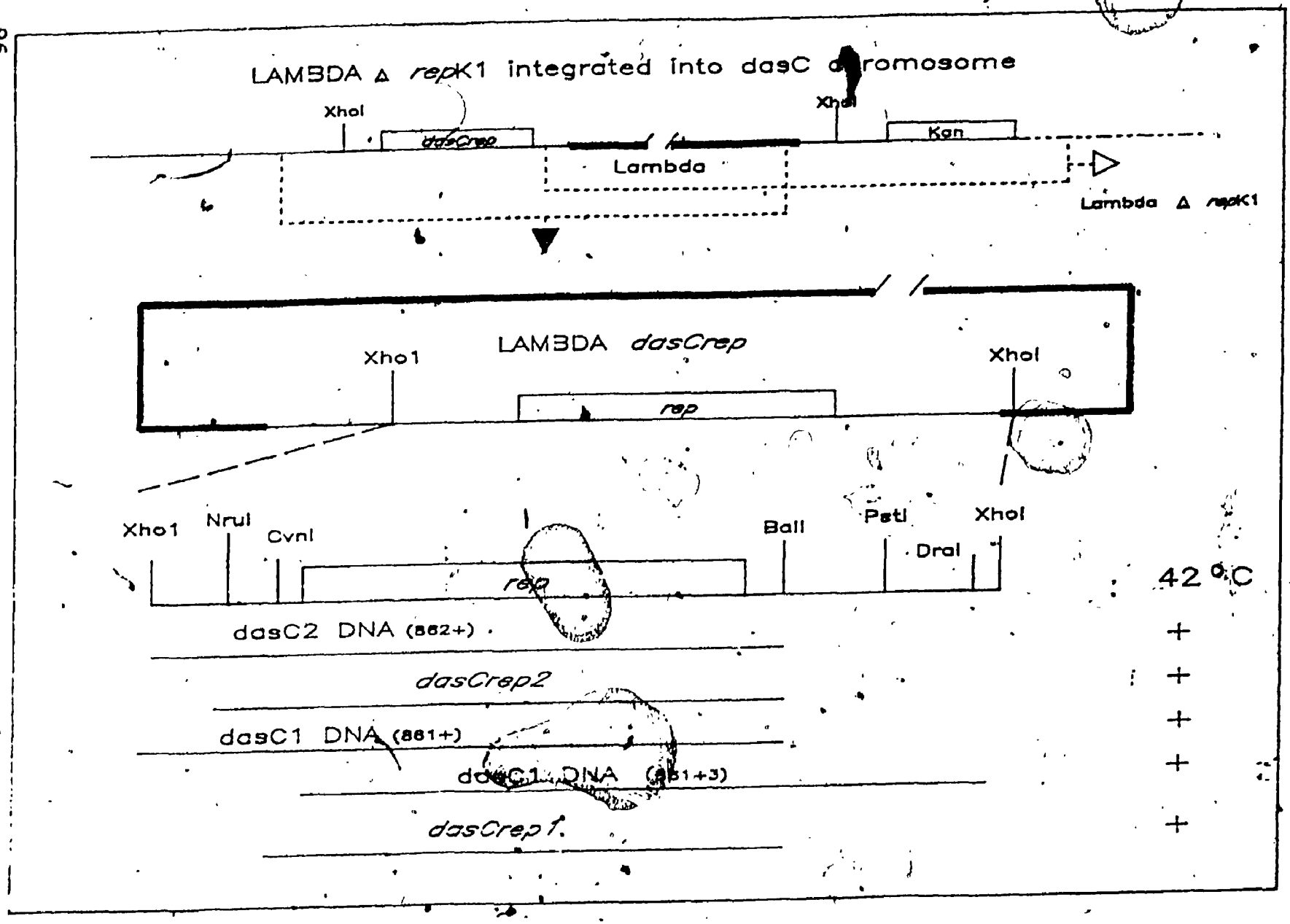
The rep gene was isolated as a lambda dasCrep construct (Fig 12) as detailed in materials and methods 2:32. The E. coli DNA insert was then subcloned into either the plasmid pBR322 or pSP65 using the XhoI site 900 bp upstream of the rep gene and the BalI site 300 bp downstream of the rep gene (plasmids pBR862+ and pSP861+). Also isolated from the TC861 (das) DNA was a DraI fragment extending from DraI (320) at the rep promoter to a second DraI site 760 bp downstream of BalI, this plasmid was named pSP861+3.

These plasmid constructs were introduced into the temperature sensitive parent strain TC182 as well as control pBR322 and pSPrep(N-P)(wild type) plasmids. All four were then tested for ability to support growth at 42°C.

The pBR322-carrying strain as expected did not grow at 42°C indicating that the presence of this plasmid will not suppress the dnaA46 mutation. The strains which carried

Fig 12. **Isolation of dasC DNA**

The induction of lambda▲repK12 TC861 and TC862 lysogens allowed isolation of lambdadasCrep phage (see text) The DNA which was subcloned onto pBR322 or pSP65 is shown in this diagram. The ability of this DNA (when supplied in trans) to permit growth at the restrictive temperature (42°C) in TC182 is scored.


LAMBDA Δ repK1 integrated into dasC chromosome
XhoI
XhoI
dasCrep
Kan
Lambda
Lambda Δ repK1
LAMBDA dasCrep
Xho1
XhoI
rep
Xho1
NruI
CvnI
BalI
PstI
XhoI
DraI
rep
42 °C
dasC2 DNA (862+)
+
dasCrep2
+
dasC1 DNA (861+)
+
dasC1 DNA (861+3)
+
dasCrep1.
+

the dasC DNA were capable of growth at the restrictive temperature of 42°C but the colony survival was less than in the TC861 and TC862 which carried this DNA (dasC) in cis. In the second control, where pSPrep(N-P) is introduced into the TC182 ts strain, a partial alleviation of the temperature sensitive phenotype was observed - the TC182/pSPrep(N-P) now being capable of surviving at 40°C, although unlike the DNA subcloned from the dasC strain it did not permit growth at 42°C.

4.06 Isolation of dasCrep

The NruI(1)-BalI(2670) fragment was isolated from pBR862+ (dasCrep2 + upstream DNA) and recloned creating plasmid pBR862 (dasCrep2). The NruI site is located 300 bp upstream of the rep promoter and the BalI site is 320 bp downstream of the rep transcription termination site. This plasmid therefore contains only a minor quantity of extraneous E. coli DNA. A similar fragment was isolated from pSP861+ (dasCrep1 + and upstream DNA) to create pSP861 (dasCrep1); also constructed was a plasmid pSP861-border which had less extraneous TC861 (das) DNA as it was subcloned using the CvnI(178)-BalI(2670) restriction sites.

These subclones were then tested for their ability to suppress the dnaA46 mutation when supplied in trans. There was no difference between these subclones and the originally

isolated DNA in terms of their ability to suppress the temperature sensitive phenotype of strain TC182 (Fig 13).

As a further control the DNA isolated from TC862(das) was reisolated and subcloned into the pSP64 plasmid in the same orientation as the DNA from TC861(das) in plasmid pSP861 and retested for its ability to supress the dnaA46 mutation when supplied in trans: Its capacity to confer the temperature resistant phenotype was unaltered.

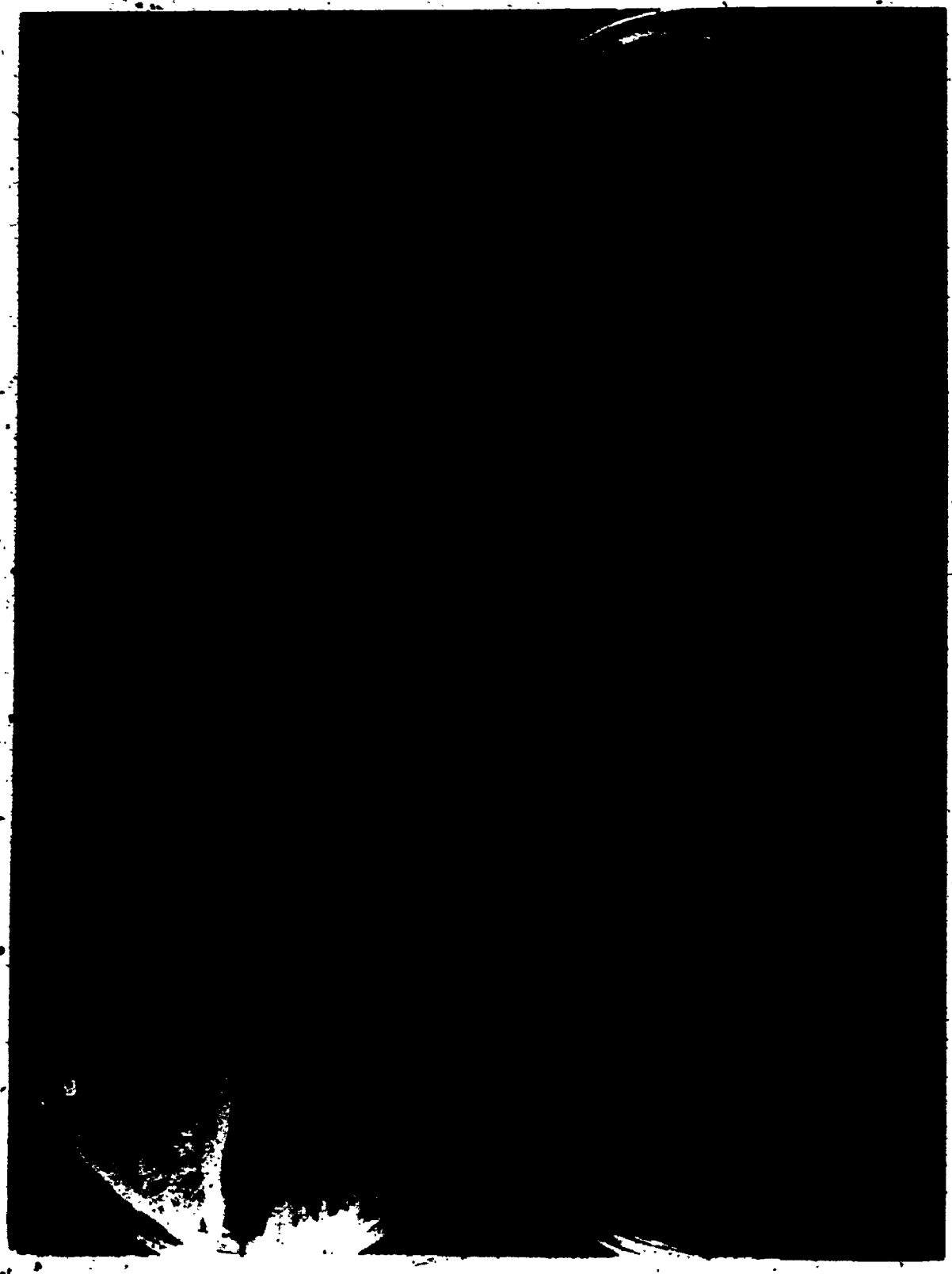
A further check was done to ensure that the above DNA contained the dasC phenotype. The dasC1 and dasC2 DNA from XhoI site (approximately 500 bp upstream from the NruI(1) of rep sequence) to the EcoRI site (452) (in the rep gene) was subcloned into the pSP65 plasmid and used to transform the TC182 dnaA46 temperature sensitive strain. These bacteria were not able to grow at the restrictive temperature.

The rep gene isolated from TC861(das) will from now on be referred to as dasCrep1; similarly rep isolated from TC862(das) will be dasCrep2.

At this point the plasmid pSPrep(N-P)(wt) was modified until only the restriction fragment corresponding to that present in pSP861(dasCrep1) remained [NruI(1)-BalI(2670)] and tested for its ability to suppress the temperature sensitive phenotype of dnaA46 when supplied in trans (Fig 13). It was discovered that this DNA could now act as a partial suppressor of the dnaA mutation Fig 13. This indicates that the fragment downstream of rep BalI(2670)-

Fig 13. **Growth of strains at the permissive and restrictive temperature**

Bacteria plates on the left were incubated at 32°C for 48 h while those on the right were incubated at 42°C. Strain TC152 (wt) is labeled A (growth at 32°C) and A'(growth at 42°C) similarly TC182 (*dnaA46* ts) is labeled B,B'; TC861 (das) C,C'; TC862 (das) D,D'; Strain TC182/pSP*rep*$^+$ a,a'; TC182/pBR862 (*dasCrep2*) b,b'; pSP861+3 (*dasCrep1*, *dasC*) c,c'; TC182/pSP861+ (*dasCrep1*) d,d'; TC182/pHBH30 (6Kb from 84.5 min *E. coli* inclusive of *rep*$^+$ and *dasC*$^+$) e,e'; TC182/ pBR322 f,f'; TC182/pSP*rep*(N-P) (*rep*$^+$,*dasC*$^+$) g,g'; TC182/pGT26 (*uvrD*$^+$) h,h'.



PstI(3170) dasC$^+$ confers an anti suppressor phenotype in wild type which it is not capable of conferring if isolated from the TC861(das)$^-$ strain (pSP861+3 dasCrep1 dasC$^-$ can suppress dnaA mutants).

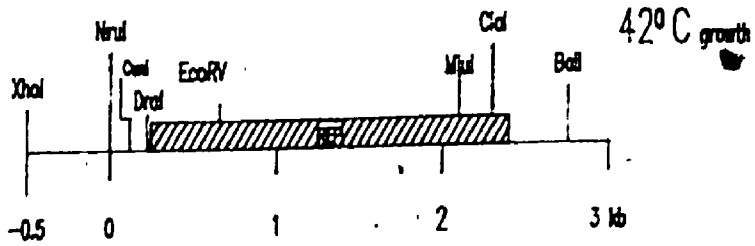
4.07 Deletions into dasCrep

To test whether the Rep protein allele is required for the temperature resistant phenotype of dasC, various rep deletions were constructed. Depicted in Fig 14 are the created subclones and the results of introducing them in trans into the TC182 dnaA46 (ts) strain.

The clones dasCrep (EV-B) which have a deletion from NruI(1) to EcoRV(686) restriction sites. This deletion removes the rep promoter and eighty nine N terminal amino acids. The resulting dasC subclones when introduced into the dnaA46 temperature-sensitive host did not allow the E. coli to grow at 42°C. The overlapping upstream dasC DNA [XhoI(-500) to EcoRI(452)] containing the rep promoter and eleven N-terminal amino acids is by itself incapable of permitting growth at 42°C. A deletion into the C-terminus of the dasCrep2 allele, between the restriction sites ClaI (2168) and BalI(2670) which results in the truncating of DasCrep by 42 amino acids, also abolished the ability of the dasCrep2 DNA to complement the temperature-sensitive phenotype. These data suggest the intact rep gene in trans is required for

Fig 14 **Deletions into rep and dasCrep alleles**

The restriction map of rep and bordering DNA shows only those restriction sites used in these experiments. The hatched box covers the rep gene. The left hand panel diagrams the dasCrep1 deletions; scored alongside is the ability of that subclone when introduced into TC182 to permit growth at 42°C. The right hand panel is organized similarly dasCrep2 and rep (wt) deletions are shown and scored.

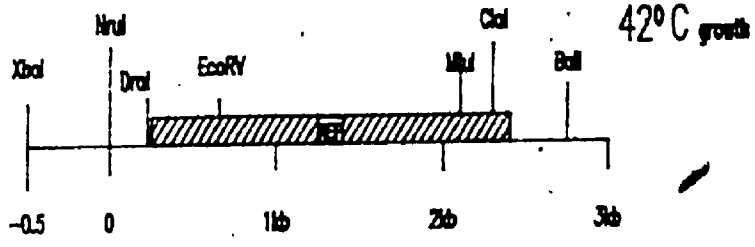


dasOrep1 (Δ border) ++++

dasOrep1 (861+3) ++++

dasOrep1 (EV-B) ----

upstream 861 DNA ----



dasOrep2 (862) ++++

C-term. del of *dasOrep2* ----

dasOrep2 (EV-B) ----

upstream 862 DNA ----

wt *rep* (N-P) ----

wt *rep* (N-B) ++++

some colony survival at 42°C dasC suppressor activity is Rep protein production.

Also introduced in trans and tested for growth at 42°C was a multicopy plasmid carrying the helicase similar to Rep the DNA Helicase II or UvrD. It was found that the uvrD gene when amplified could suppress the dnaA46 mutation.

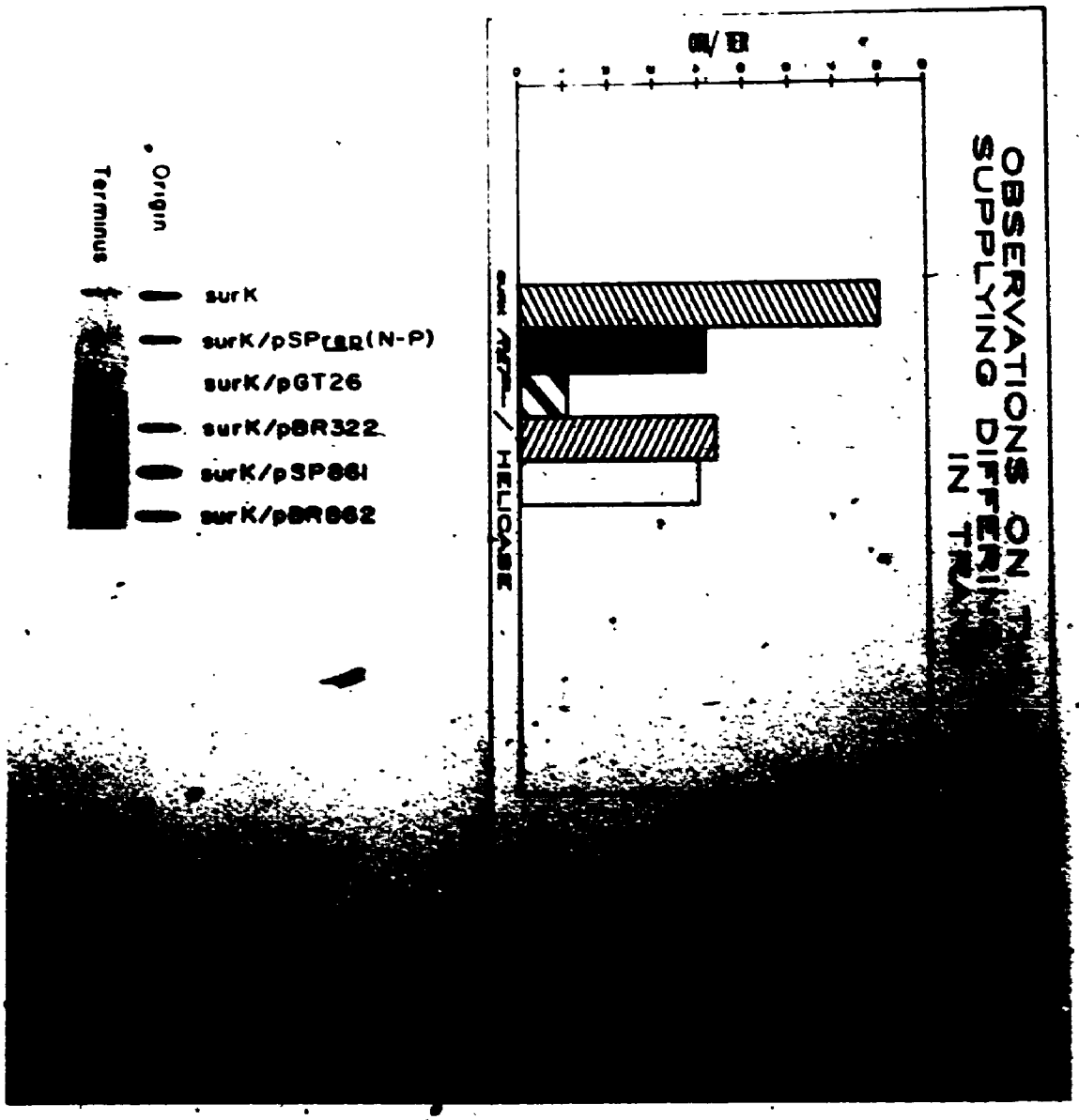
4.08 The effect of introducing dasCrep alleles into a rep⁻ strain

As observed previously, the TC862 and TC861 strains carrying the dasCrep2 and dasCrep1 genes, respectively, had no alteration in origin/terminus ratio. It was possible that the presence of these alleles in a dnaA46 background was obscuring their effect, therefor to observe this function of dasCrep1, dasCrep2 and compare it with wild type rep these genes were introduced into the rep⁻ strain and their capacity to alter the origin/terminus ratio compared. The wild type Rep, as expected, reduced the origin/terminus ratio to normal levels; that the DasCrep proteins did so also indicated that this function was unaltered (Fig 14).

The presence of the plasmid carrying the uvrD gene reduced the origin/terminus ratio to below that of wild type cells (Fig 14).

Fig 15 **The effect on the origin/terminus ratio in surK*rep*⁻ cells when helicase alleles are supplied in trans**

The upper panel graphs the origin/terminus ratio in surK⁻ (Δ*rep*) strains with differing *rep* or *uvrD* alleles supplied in trans. The data was obtained by densitometary scanning the slot blots in the lower panel which show the quantity of origin or terminus ^{32}P-labelled probe hybridizing to equal quantities of genomic DNA isolated from the surK strains (the DNA was applied in three differing dilutions (not shown) in order to be certain the result was quantitative).


Origin
Terminus
surK
surK/pSP
surK/pGT26
surK/pBR322
surK/pSP861
surK/pBR862
HELICASE
OBSERVATIONS ON
SUPPLYING DIFFERI
IN TR

4.09 Sequence of the dasCrep genes

The dasCrep alleles were sequenced in one strand in order to compare them to wild type. The sequence of the alleles are detailed in Figure 16. There appear to be no differences between dasCrep and rep.

4.10 The interaction of dasCrep and rep with mutations in dnaA

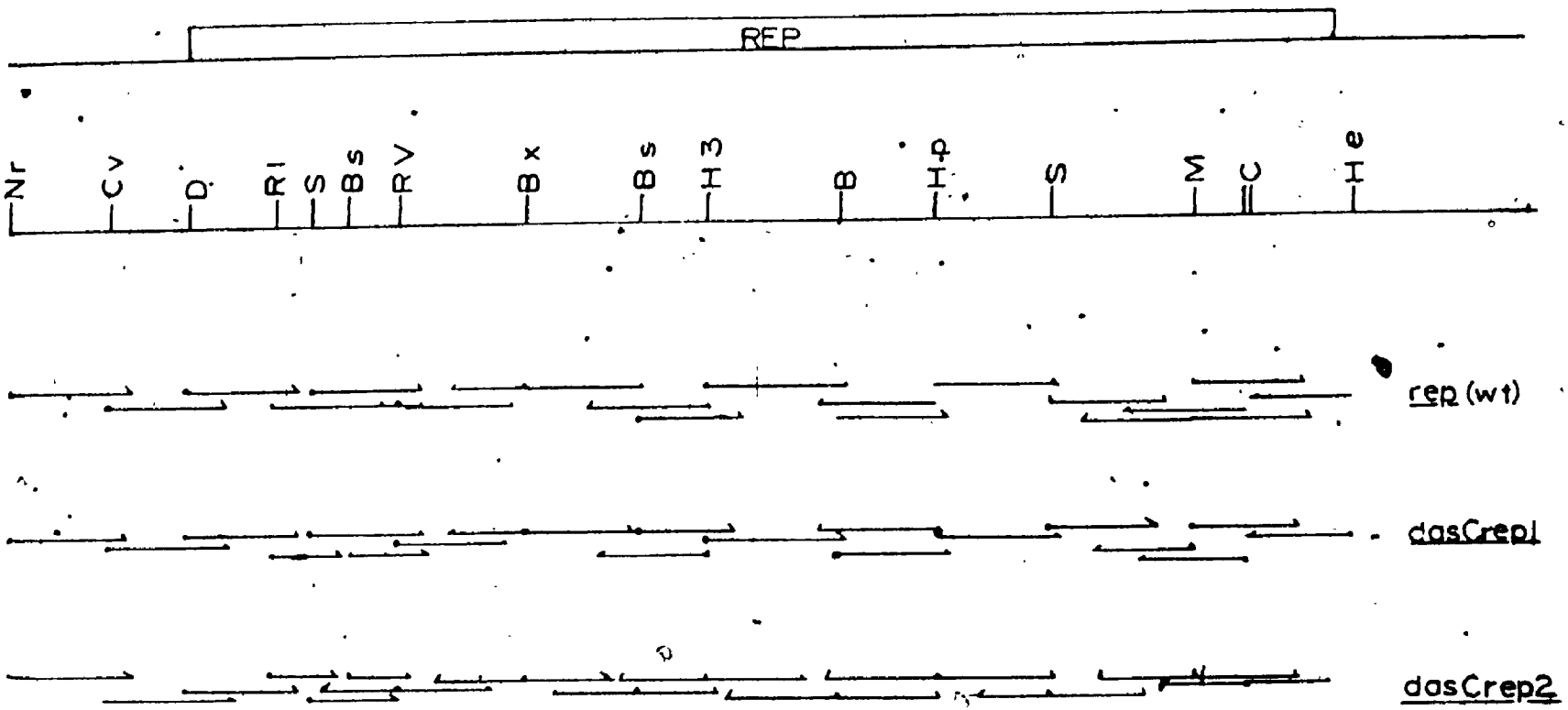
There are two simple interpretations of the suppressor activity in dasCrep and rep. The first is that Rep without the anti suppressor's presence can bypasses the cell's ordinary requirement for the DnaA protein, obliterating the problem of the mutated protein. The second possibility is that in the absence of the antisuppressor the Rep protein can interact with the mutated DnaA and restore its function either by restoring stability or by changing its conformation to closer to wild type.

To distinguish between these two possibilities we examined the effect of introducing dasCrep into the NS388 strain (Schaus et al., 1981). This strain has a temperature-sensitive tRNA gene supF81 which encodes a suppressor tRNA which allows read-through of the amber nonsense codon in the dnaA366 gene which otherwise leads to the production of a truncated nonfunctional DnaA. If DasCRep bypassed the

Fig 16 **Sequence of the rep alleles**

The region shown is the section of E. coli sequenced; the box covers the rep gene. The line indicates the restriction sites used to construct M13rep subclones restriction endonucleases are designated respectively Nr, NruI; Cv, CvnI; D, DraI; RI, EcoRI; S, SstII; B, BssHII; Bs, ;B, BstEII RV, EcoRV; Bx, BstXI; H3, HindIII; Hp, HpaI; M, MluI; C, ClaI; Hc, HaeII.

The continuous arrows indicate the direction of dideoxy sequencing; their length is proportional to the amount of sequence determined from an individual clone. Three sets of lines are indicated representing the sequence from rep (wt); dasCrep1, and dasCrep2.


REP
Nr
Cv
D
RI
S
Bs
RV
Bx
Bs
I3
B
Ip
S
Σ
C
Ie
rep (wt)
dasCrep1
dasCrep2

requirement for DnaA protein the introduction of this allele would allow growth at 42°C.

The results indicate that the presence of the mutated DnaA protein is necessary for proper suppression by dasC. Introduction of the dasCrep1 and dasCrep2 allele does not allow growth at 42°C in these strains.

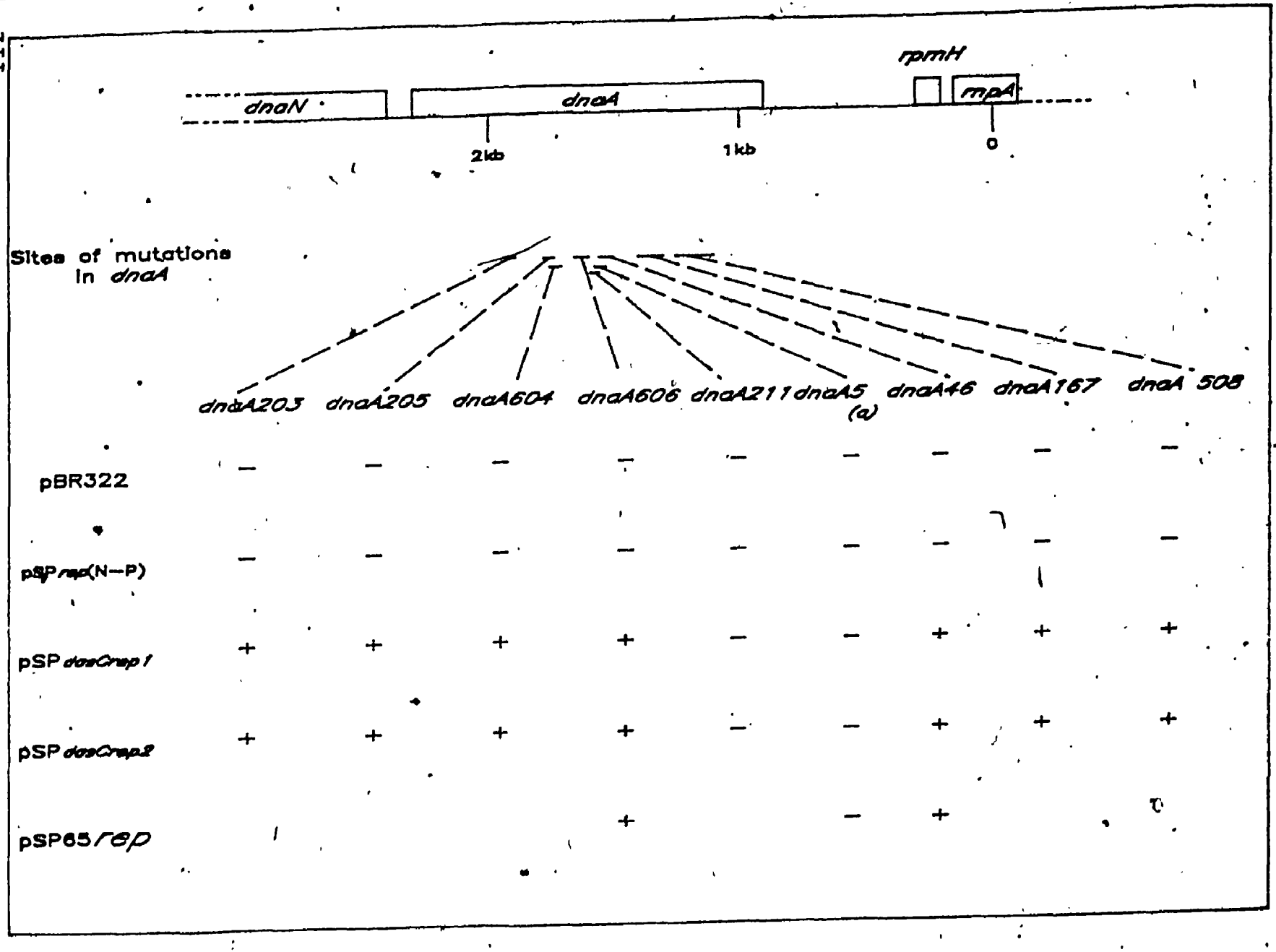
The introduction of the DNA Helicase II protein on a multicopy plasmid into the NS388 strain indicated that while this protein was capable of acting as a suppressor of the dnaA mutations it achieved this affect by another mechanism. It bypassed the cells requirement for the DnaA protein and, when supplied in trans, allowed growth at 42°C in the NS388 strain. These results were confirmed by the use of a similar second dnaA/ts tRNA combination strain NS387 dnaA311(Am) ,supF81(Ts)

4.11 The rep alleles can suppress other dnaA ts mutants

It has been noted with other extragenic suppressors of dnaA that there is a clustering in the lesions in the dnaA which can be suppressed, perhaps indicating the location of the protein domain with which they interact. I tested a wide range of dnaA mutants located in differing regions of dnaA (Hansen et al., 1984) for their suppressibility by the dasCrep and rep$^+$ genes (Fig 17). The dasC genes appeared to

Fig 17 **Interaction of the *rep* alleles supplied in trans to differing *dnaA* mutants**

The data on the position of the *dnaA* mutants has been obtained from Hansen and von Meyenberg (1984). The table scores the ability of the plasmids indicated to permit growth at the restrictive temperature. The (a) notation indicates that the restrictive temperature used for this strain, PC5 which was isolated by Carl (1970), was 37°C (as indicated by the positive control pGT26 when supplied in trans)


rpmH
dnaN
dnaA
rnpA
2kb
1kb
0
Sites of mutations in dnaA
dnaA203
dnaA205
dnaA604
dnaA606
dnaA211
dnaA5 (a)
dnaA46
dnaA167
dnaA 508
pBR322
pSP rep(N–P)
pSP dnaCrep1
pSP dnaCrep2
pSP65 rep
– – – – – – – – –
– – – – – – – – –
+ + + + – – + + +
+ + + + – – + + +
+ – + +

effectively suppress any mutations in the N and C terminals of dnaA but there is a region close to the centre of the dnaA in which mutations are not capable of being suppressed by the dasCrep or rep genes.

4.12 DISCUSSION

One approach utilized in studying a complicated system is the genetic one of isolating a series of extragenic suppressors. The products so identified either interact with the original protein studied or bypass it using another pathway.

This approach was used to study the DnaA gene and has led to the isolation of various das (dnaA suppresor) mutations. Two methods of altering the suppressor gene have been determined; the first, typified by that in the rpoB gene (encoding the β-subunit of RNA polymerase); is a mutation in the suppressor gene presumably altering it so that it interacts differently with other cellular proteins (Atlung et al., 1981). The second type of suppressor alteration, typified by the groE operon, is an over expression of the gene products achieved by an amplification of the suppressor gene - an affect that can be mimicked by introducing this gene into the cell on a multicopy plasmid. The two genes encoded by the groE operon are groES and groEL. They suppress lesions in a process that requires the presence of the DnaA protein, possibly interacting with it. The dnaA lesions they can suppress are clustered in the centre of the primary part of the amino acid sequence (Jenkins et al, 1986; Fayet et al., 1986).

To determine which category the dasC suppressor falls in, rep's level of expression in the TC strains was examined. The quantity of Rep in the original wild type cell and the derived dnaA46 temperature-sensitive strain was compared to that of the dasC derivatives. No change was observed.

However the sequencing of the dasCrep alleles indicates that they have not been mutated in the rep coding sequence analogously to the rpoB suppressor mutations. The alteration in the E. coli genome which allows suppression of the dnaA46 mutation is an alteration of the DNA downstream of rep. This DNA appears to have a dnaA antisuppressor activity which will be referred to as dasC.

The dasCrep$^+$ and rep$^+$ alleles as well as the combination of rep plus dasC$^+$ were supplied in trans to an array of dnaA mutations which had been mapped in the dnaA gene (Hansen et al., 1984). It was determined that unlike the rpoB and groE suppressors rep suppresses the lesions in the N and C terminus of dnaA but is ineffective at allowing colony formation at 42°C in strains carrying the dnaA5 and dnaA211 allele in the centre of the gene.

There are two possible routes for achieving extragenic suppression. The first model 1 (Fig 18) suggests the target (DnaA) and the suppressor (Rep) interact leading to an increased stability of the DnaA or increased activity due to

the new suppressor/DnaA46 combination returning the conformation of DnaA closer to wild type and it regains activity.

The second model (model 2) suggests that the suppressor bypasses the cells normal requirement for the DnaA protein and uses a substitute pathway.

In order to determine the method by which the rep suppressor acts this DNA was introduced into a temperature sensitive NS388 strain carrying dnaA366 and supF81(ts) mutations. At 32°C the tRNA encoded by the supF81 gene allows read-through of the nonsense codon in the dnaA gene. At higher temperatures the supF81 gene is no longer active and as DnaA is not produced no growth occurs (Schaus et al., 1981).

If suppression is achieved by model 1 the suppressor will not be capable of interacting with the truncated peptide and no suppression should take place.

The rep suppressor fits the first model.

In order to compare the suppressive nature of the dasC with the effect of introducing another helicase into the dnaA system the effect of introducing the similar UvrD (DNA Helicase II) was studied. It was found that although no suppressors of dnaA had been mapped to 85 min on the E. coli chromosome (the uvrD locus) the amplification of this gene on a multicopy plasmid allowed it to act as a suppressor. This suppressor acts in accordance to model 2, bypassing the cells normal requirement for DnaA.

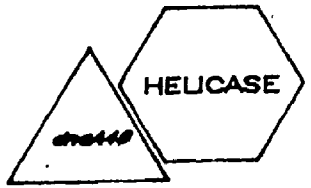
Fig 18 **Models of the possible method of suppressor action**

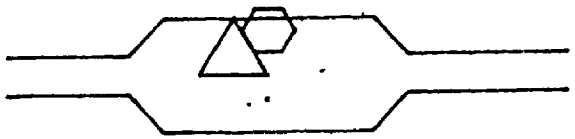
This diagram shows the possible consequences of different methods of suppressor interaction in two dnaA mutants.

Model 1: Direct interaction between the DnaA protein and the helicase.

Model 2: Bypass of DnaA requirement of the cell by the helicase.

MODEL 1

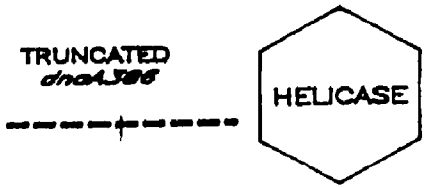


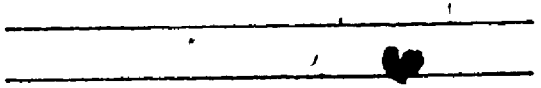


INITIATION AT ORI C

SUPPRESSION AT 42 ° C

TRUNCATED *dnaA366*

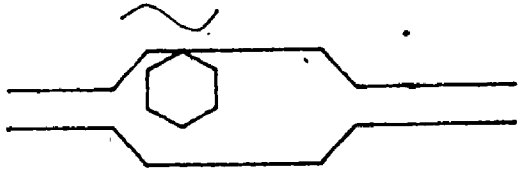




NO INITIATION

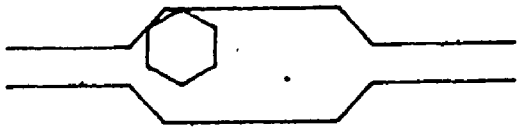
NO SUPPRESSION

MODEL 2



SUPPRESSION AT 42 ° C

TRUNCATED *dnaA366*



SUPPRESSION AT 42 ° C

It is possible that DNA Helicase II in high quantities leads to nonspecific unwinding of the DNA helix at oriC and this allows initiation to proceed. The possibility that the DnaA protein appears to interact with the Rep helicase suggests that dasC may have some role in regulating a specific unwinding process utilizing Rep.

In order to investigate the interaction of Rep and UvrD at the origin, the rate of initiation in a rep host was examined when the two DNA helicases were supplied in trans. It had been noted that there is a change in the timing of initiation in rep hosts compared to wild type, further that when Rep was supplied in trans the timing reverted wild type levels (Lane and Denhardt, 1975; Colasanti and Denhardt, 1987). The overproduction of Rep due to it's coding sequences being under the control of the lambda P_L promoter does not reduce this origin/terminus DNA ratio below wild type levels (Colasanti and Denhardt, 1987).

The dasCrep gene supplied in trans appeared to act similarly to wild type rep; it reduced the ratio of initiation to termination of chromosomal DNA to normal. Surprisingly, the introduction of uvrD led to an even greater than normal decrease. This again allows the postulate that this DNA helicase is nonspecifically unwinding the chromosome, possibly allowing the replication fork to progress at a faster than usual rate. With less replication in progress

at any given time the quantity of origin DNA is more comparable to the quantity of terminus DNA.

The dasC phenotype may, possibly, be due to a lesion in the BalI(2670)-PstI(3170) fragment (dasC) perturbing a regulatory system of the Rep protein. This effect can be mimicked with low efficiency by supplying the rep gene in trans on a multi copy plasmid.

There are two simple models for this system's mode of action. Model 3 proposes that the dasC DNA encoded a protein which normally interacts with Rep negatively regulating any involvement of Rep with initiation of DNA replication. The second possibility is that the dasC DNA binds a positively regulatory protein which allows Rep to interact with DnaA in initiation of DNA replication. The sequencing of the dasC DNA is in progress and will be searched for an open reading frame which could encode an regulatory protein.

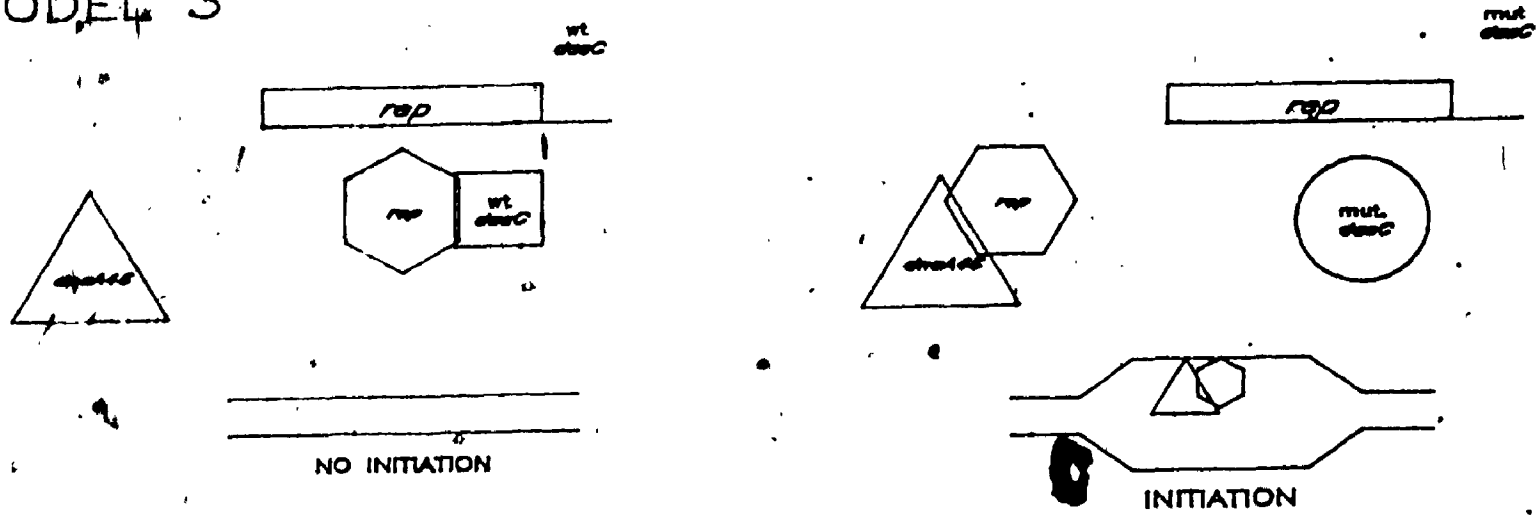
The dasC DNA appears to act in a manner similar to the trxA gene also thought to be the site of the dasC anti-suppressor (T. R. Hupp and J. M. Kaguni, Abstracts from 1987 September meeting at Cold Spring Harbour). The trxA gene like our dasC maps between the rep and rho genes but is, however, 1.5 kb downstream of the PstI site (Lim et al., 1985) which borders our dasC DNA. The fact that Hupp and Kaguni's anti suppressor activity requires the expression of the TrxA protein, further distinguishes it from the DNA isolated by us as dasC. That Hupp and

Fig 19 **Models of Rep, DnaA and dasC interaction**

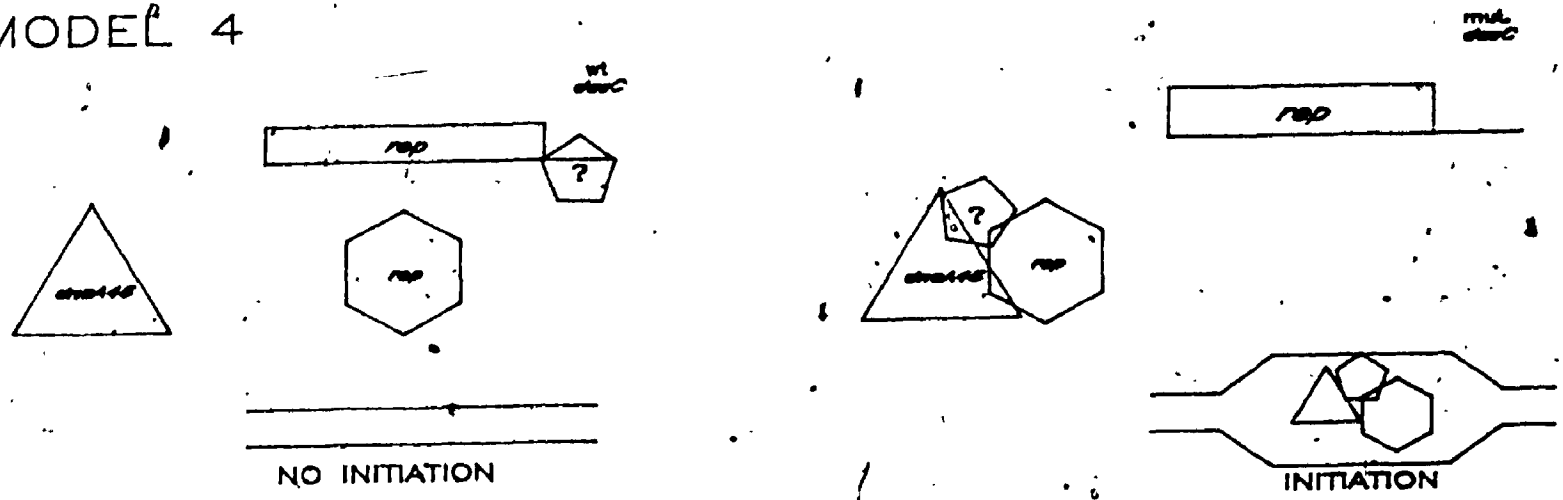
Model 3 : If dasC encodes a negatively regulating protein which binds to Rep. A lesion in dasC could abolish this protein's production and allow DnaA-Rep interaction

Model 4 : If the region of DNA designated dasC is a binding site for a protein necessary for utilization of Rep in the initiation process. An alteration of the dasC DNA such that this putative protein (?) could no longer bind and would be left free to interact with DnaA and Rep.

MODEL 3



MODEL 4



Kaguni's suppressor resides in sequence bordering the trxA gene.

The dasC suppressor mutation is a lesion in the "dasC" DNA removing a regulator of Rep allowing this helicase to interact with the DnaA protein and thus initiation to take place.

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2. Gilchrist, C.A., and Denhardt, D.T. (1987) Escherichia coli rep gene: Sequence of the gene, the enclosed helicase, and its homology with uvrD. Nucleic Acids Research, Vol. 15, p. 465-475

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1. Canadian Federation of Biological Societies Meeting, June 16-20, 1986, Guelph, Ontario. "Sequences of the rep gene and protein." C.A. Gilchrist and D.T. Denhardt.

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